

11-20-00

PATENT

A

Attorney Docket No. GENENT.68A2D1

Date: November 16, 2000

Page 1

ASSISTANT COMMISSIONER FOR PATENTS

WASHINGTON, D.C. 20231

ATTENTION: BOX PATENT APPLICATION

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): Jin, et al.

For: EFFECTS OF IFN- $\gamma$  ON CARDIAC HYPERTROPHY

Enclosed are:

- (X) 9 sheet(s) of drawings.
- (X) A copy of an Associate Power of Attorney.
- (X) This application is a Divisional of prior parent application 09/273,099 filed March 19, 1999, based on provisional application Serial No. 60/080,448 filed on April 2, 1998.
- (X) A copy of Declaration and Power of Attorney from prior application is enclosed.
- (X) Incorporation by Reference. The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
- (X) Return prepaid postcard.
- (X) PRELIMINARY AMENDMENT is enclosed. Prior to calculating the filing fee, please enter the Preliminary Amendment

## CLAIMS AS FILED

FOR	NUMBER FILED	NUMBER EXTRA	RATE	FEE
Basic Fee			\$710	\$710
Total Claims	31 - 20 =	11 ×	\$18	\$198
Independent Claims	4 - 3 =	1 ×	\$80	\$80
If application contains any multiple dependent claims(s), then add			\$270	\$0
<b>TOTAL FILING FEE</b>				<b>\$988.00</b>

- (x) A check in the amount of \$988.00 to cover the filing fee is enclosed.
- (X) The Commissioner is hereby authorized to charge any additional fees which may be required, now or in the future, or credit any overpayment to Account No. 11-1410. A duplicate copy of this sheet is enclosed.

JC871 U.S. PTO  
 11/16/00

JC862 U.S. PTO  
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09/273,099  
 60/080,448

PATENT

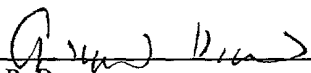
Attorney Docket No. GENENT.68A2D1

Date: November 16, 2000

Page 2

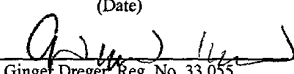
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(X) Please use Customer No. 20,995 for the correspondence address.

  
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Ginger R. Dreger  
Registration No. 33,055  
Attorney of Record

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## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Jin, et al.	)	Group Art Unit 1647
			)	
Appl. No.	:	Unknown	)	I hereby certify that this correspondence and all
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Filed	:	Herewith	)	the United States Postal Service as first-class
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For	:	EFFECTS OF IFN- $\gamma$ ON CARDIAC	)	Commissioner for Patents, Washington, D.C.
		HYPERTROPHY	)	20231, on
			)	
			)	November 16, 2000
			)	(Date)
			)	
Examiner	:	FITZGERALD, D.	)	Ginger Dreger, Reg. No. 33,055

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

This Preliminary Amendment is filed concurrently with the filing of a divisional patent application claiming priority to parent application No. 09/273,099 filed March 19, 1999, based on provisional application Serial No. 60/080,448 filed on April 2, 1998.

Please amend this application as follows.

In the Specification:

On page 1, after the title, please insert the following sentence: --This is a continuation of application Serial No. 09/273,099 filed on March 19, 1999, which claims priority of provisional application Serial No. 60/080,448 filed on April 2, 1998.--

On page 1, line 30, after "infection," insert --but--.

On page 2, line 5, after "IFN- $\beta$ )" insert a comma; in line 6, change "PH" to --pH--; in line 8, after "is" insert --the--; in line 10, delete the comma (first occurrence); in line 14, change "E. coli derived" to --*E. coli*-derived--; in line 22, change "disfunction" to --dysfunction--.

On page 3, line 5, change "Wilet" to --Wiley--; in line 9, after "as" (second occurrence) insert --of--.

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Filed : Herewith

On page 4, line 15, change "non-myocyte derived" to --non-myocyte-derived--; in line 16, delete the comma (first occurrence); in line 23, change "short term" to --short-term--.

On page 5, line 24, after fosinopril," insert --and--; in line 26, after "acetazolamide," after "indapamide;" and after "verapamil," insert --and--.

On page 6, line 22, change "agent" to --agents--.

On page 7, line 7, change "Figure 1" to --Figures 1A-1F--; in line 12, after "respectively." insert --D, E, F--; in line 13, change "occurrence" to --occurrence: control, PGF<sub>2α</sub>, and PGF<sub>2α</sub>+IFN-γ, respectively--; in line 18, change "Figure 2" to --Figures 2A-2E--; in line 29, change "Figure 3" to --Figures 3A-3F--.

On page 8, line 2, after "respectively." insert --D, E, F--; in line 3, change "occurrence" to --occurrence: control, PE, and PE+IFN-γ, respectively--; in line 8, change "Figure 4" to --Figures 4A-4C-- and change "cardiac hypertrophy" to --the ratio of heart weight, ventricular weight, and left ventricular weight to body weight, respectively,--; in line 14, change "Figure 5" to --Figures 5A-5B-- and change "HR" to --HR, respectively--; in line 19, change "Figure 6" to --Figures 6A-6D--; in line 25, change "Figure 7" to --Figures 7A-7C-- and change "weight" to --weight, respectively,--; in line 31, change "Figure 8" to --Figures 8A-8C--; in line 32, change "weight" to --weight, respectively,--.

On page 9, line 6, change "Figure 9" to --Figures 9A-9C--; in line 7, change "pressure" (first occurrence to --pressure, respectively,--; in line 27, change "will become" to --becoming--; in line 30, change "range," to --range;--.

On page 10, line 5, change "(Genentech" to --(Genentech--; in line 9, change "and" to --are--; in line 23, change "myrofibrils" to --myofibrils--; in line 26, change "a" to --as--.

On page 12, line 27, after "is" insert --the--.

On page 14, line 3, change "bodyweight" to --body weight--; on line 15, change "b." to --c.--; in line 21, after "IFN-γ" insert a comma; in line 30, change "of" (first occurrence) to --for--.

On page 15, line 6, change "death" to --deaths--; in line 12, change "high dose" to --high-dose--; in line 24, change "resulted" to --result--; in line 26, after "metoprolol," insert --or--; in line 27, after "fosinopril," insert --or--; in line 29, after "acetazolamide," insert --or--; in line 30, after "verapamil," insert --or--; in line 31, change "here" to "herein--.

On page 16, line 30, after "4,499,014" insert a comma.

On page 17, line 24, change "Actimmune" to --Actimmune® rhuIFN-γ-1b--.

Appl. No. : Unknown  
Filed : Herewith

On page 19, line 7, after "0.75" insert --%--; in line 22, after "purity" insert --standards--.

On page 20, line 5, change "released" to --release--; in line 9, delete the ")" after "methacrylate".

On page 21, line 6, change "0.5 m,<sup>2</sup>" to --0.5 m<sup>2</sup>--; in line 23, after "metoprolol," insert --or-- and after "difenidipine," insert --or--; in line 25, after "verapamil," insert --or--; in line 27, after "acetazolamide," insert --or--; in line 28, after "fosinopril," insert --or--.

On page 22, line 27, change "rod shaped" to --rod-shaped--; in line 31, change "myocyte" to --myocytes--.

On page 23, line 9, change "rod shaped" to --rod-shaped--; in line 19, change "PGF<sub>2α</sub> induced" to --PGF<sub>2α</sub> induced--; in line 20, after "cytokines" and after "IFN-β" insert commas.

On page 24, line 1, after "light" insert a hyphen; in line 2, change "temperature controlled" to --temperature-controlled--; in line 12, change "intraperitoreal" to --intraperitoneal--; in line 27, change "3 cm" to --3-cm--.

On page 25, line 9, change "One way" to --One-way--; in line 11, after "using" insert --the--.

On page 26, line 13, after "GAPDH" insert a comma; in line 15, after "60°" insert --c--.

On page 27, line 4, insert a hyphen between "fluprostenol" and "induced"; in line 7, change "IFN-γ and vehicle treated" to --IFN-γ- and vehicle-treated--; in line 11, insert a hyphen between "vehicle" and "treated"; in line 17, insert a hyphen between "fluprostenol" and "induced"; in line 31, delete the comma.

On page 28, line 9, change the semi-colon to a comma and insert a comma after "collagen I"; in line 12, change "cardia" to --cardiac--.

#### In the Claims:

Please cancel claim 1-28.

Please add the following claims:

--29. A method of treating cardiac hypertrophy in a patient comprising administering to the patient a therapeutically effective amount of interferon gamma (IFN-γ), wherein said cardiac hypertrophy is selected from the group consisting of post myocardial infarction hypertrophy, hypertrophy associated with hypertension, aortic stenosis, valvular regurgitation, cardiac shunt and congestive failure.

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30. The method of claim 29 wherein said patient is human.
31. The method of claim 30 wherein said IFN- $\gamma$  is recombinant human IFN- $\gamma$  (rh-IFN- $\gamma$ ).
32. The method of claim 31 wherein said IFN- $\gamma$  is rhIFN- $\gamma$ -1b.
33. The method of claim 30 wherein said cardiac hypertrophy has been induced by myocardial infarction.
34. The method of claim 33 wherein said IFN- $\gamma$  administration is initiated within 48 hours following myocardial infarction.
35. The method of claim 34 wherein said IFN- $\gamma$  administration is initiated within 24 hours following myocardial infarction.
36. The method of claim 30 wherein said patient is at risk of developing cardiac hypertrophy.
37. The method of claim 36 wherein said patient has suffered myocardial infarction.
38. The method of claim 37 wherein said IFN- $\gamma$  administration is initiated within 48 hours following myocardial infarction.
39. The method of claim 38 wherein said IFN- $\gamma$  administration is initiated within 24 hours following myocardial infarction.
40. The method of claim 30 wherein said IFN- $\gamma$  is administered in combination with at least one further therapeutic agent used for the treatment of cardiac hypertrophy or a heart disease resulting in cardiac hypertrophy.
41. The method of claim 40 wherein said further therapeutic agent is selected from the group consisting of  $\beta$ -adrenergic-blocking agents, verapamil, diltiazem, and diltiazem.
42. The method of claim 41 wherein said  $\beta$ -adrenergic-blocking agent is a carvedilol, propranolol, metoprolol, timolol, exprenolol or tertatolol.
43. The method of claim 40 wherein said IFN- $\gamma$  is administered in combination with an antihypertensive drug.
44. The method of claim 40 wherein said IFN- $\gamma$  is administered with an ACE-inhibitor.
45. The method of claim 40 wherein said IFN- $\gamma$  is administered with an endothelin receptor antagonist.

**Appl. No.** : **Unknown**  
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46. The method of claim 40 wherein said IFN- $\gamma$  is administered following the administration of a thrombolytic agent.

47. The method of claim 45 wherein said thrombolytic agent is recombinant human tissue plasminogen activator (rht-PA).

48. The method of claim 40 wherein said IFN- $\gamma$  is administered following primary angioplasty for the treatment of acute myocardial infarction.

49. A method for making a pharmaceutical composition for the treatment of cardiac hypertrophy in a patient, wherein said cardiac hypertrophy is selected from the group consisting of post myocardial infarction hypertrophy, hypertrophy associated with hypertension, aortic stenosis, valvular regurgitation, cardiac shunt and congestive failure, comprising admixing a therapeutically effective amount of interferon gamma (IFN- $\gamma$ ) with a pharmaceutically acceptable carrier.

50. The method of claim 49 wherein said pharmaceutical composition is liquid.

51. The method of claim 49 wherein said pharmaceutical composition comprises a preservative.

52. The method of claim 50 wherein said pharmaceutical composition is an injectable formulation.

53. A pharmaceutical product for use in the method of claim 29 comprising:

(a) a pharmaceutical composition comprising at least one therapeutically effective dosage of interferon gamma (IFN- $\gamma$ );

(b) a container containing said pharmaceutical composition; and

(c) a label affixed to said container, or a package insert included in said pharmaceutical product referring to the use of said IFN- $\gamma$  in the treatment of cardiac hypertrophy, wherein said cardiac hypertrophy results from an increase in myocyte cell size and contractile protein content without concomitant cell division and is characterized by the presence of an elevated level of PGF<sub>2 $\alpha$</sub> .

54. The pharmaceutical product of claim 53, wherein said container has a sterile access port.

55. The pharmaceutical product of claim 53 wherein said container is an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Appl. No. : Unknown  
Filed : Herewith

56. A method of treating cardiac hypertrophy in a patient wherein the cardiac hypertrophy has been induced by a cardiac disease other than hypertrophic cardiomyopathy of viral origin, characterized by the presence of an elevated level of  $\text{PGF}_{2\alpha}$  comprising administering to the patient a therapeutically effective amount of interferon gamma ( $\text{IFN-}\gamma$ ).

57. The method of claim 56 wherein the cardiac disease is myocardial infarction.

58. A method of treating cardiac hypertrophy in a patient wherein the cardiac hypertrophy has been induced by myocardial infarction and is characterized by the presence of an elevated level of  $\text{PGF}_{2\alpha}$  comprising administering to the patient a therapeutically effective amount of interferon gamma ( $\text{IFN-}\gamma$ ).

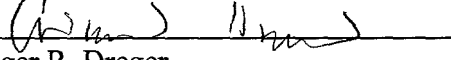
59. The method of claim 58 wherein said  $\text{IFN-}\gamma$  is initiated within 48 hours following myocardial infarction.--

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: November 16, 2000

By:   
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EXPRESS MAIL NO.: EM 168 882 624 USMAILED ON: MARCH 19, 1999**EFFECTS OF IFN- $\gamma$  ON CARDIAC HYPERTROPHY****FIELD OF THE INVENTION**

The present invention relates generally to the effects of IFN- $\gamma$  on cardiac hypertrophy. More particularly, the invention concerns the use of IFN- $\gamma$  for the prevention and treatment of cardiac hypertrophy and associated pathological conditions.

**RELATED APPLICATION**

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60\080,448 filed 2 April 1998, the contents of which are incorporated herein by reference.

**BACKGROUND OF THE INVENTION****Interferon-gamma (IFN- $\gamma$ )**

Interferons are relatively small, single-chain glycoproteins released by cells invaded by viruses or certain other substances. Interferons are presently grouped into three major classes, designated leukocyte interferon (interferon-alpha,  $\alpha$ -interferon, IFN- $\alpha$ ), fibroblast interferon (interferon-beta,  $\beta$ -interferon, IFN- $\beta$ ), and immune interferon (interferon-gamma,  $\gamma$ -interferon, IFN- $\gamma$ ). In response to viral infection, lymphocytes synthesize primarily  $\alpha$ -interferon (along with a lesser amount of a distinct interferon species, commonly referred to as omega interferon), while infection of fibroblasts usually induces  $\beta$ -interferon.  $\alpha$ - and  $\beta$ -interferons share about 20-30 percent amino acid sequence homology. The gene for human IFN- $\beta$  lacks introns, and encodes a protein possessing 29% amino acid sequence identity with human IFN- $\alpha$ 1, suggesting that IFN- $\alpha$  and IFN- $\beta$  genes have evolved from a common ancestor (Taniguchi *et al.*, *Nature* **285**, 547-549 [1980]). By contrast, IFN- $\gamma$  is not induced by viral infection, rather, is synthesized by lymphocytes in response to mitogens, and is scarcely related to the other two types of interferons in amino acid sequence. Interferons- $\alpha$  and - $\beta$  are known to induce MHC Class I antigens, while IFN- $\gamma$  induces MHC Class II antigen expression, and also increases the efficiency with which target cells present viral peptide in association with MHC Class I molecules for recognition by cytotoxic T cells.

IFN- $\gamma$  is a member of the interferon family, which exhibits the antiviral and anti-proliferative properties characteristic of interferons- $\alpha$  and - $\beta$  (IFN- $\alpha$  and IFN- $\beta$ ) but, in contrast to those interferons, is PH 2 labile. IFN- $\gamma$  was originally produced upon mitogenic induction of lymphocytes. The recombinant production of human IFN- $\gamma$  was first reported by Gray, Goeddel and co-workers (Gray *et al.*, Nature **295**, 503-508 [1982]), and is subject of U.S. Patent Nos. 4,762,791, 4,929,544, 4,727,138, 4,925,793, 4,855,238, 5,582,824, 5,096,705, 5,574,137, and 5,595,888. The recombinant human IFN- $\gamma$  of Gray and Goeddel as produced in *E. coli*, consisted of 146 amino acids, the N-terminal position of the molecule commencing with the sequence CysTyrCys. It has later been found that the native human IFN- $\gamma$  (i.e., that arising from mitogen induction of human peripheral blood lymphocytes and subsequent purification) is a polypeptide which lacks the CysTyrCys N-terminus assigned by Gray *et al.*, *supra*. More recently, the crystal structure of *E. coli* derived recombinant human IFN- $\gamma$  (rhIFN- $\gamma$ ) was determined (Ealick *et al.*, Science **252**, 698-702 [1991]), showing that the protein exists as a tightly intertwined non-covalent homodimer, in which the two identical polypeptide chains are oriented in an antiparallel manner.

IFN- $\gamma$  is known to exhibit a broad range of biological activities, including antitumor, antimicrobial and immunoregulatory activities. A particular form of recombinant human IFN- $\gamma$  (rhIFN- $\gamma$ -1b, Actimmune®, Genentech, Inc. South San Francisco, California) is commercially available as an immunomodulatory drug for the treatment of chronic granulomatous disease characterized by severe, recurrent infections of the skin, lymph nodes, liver, lungs, and bones due to phagocyte dysfunction (Baehner, R.L., Pediatric Pathol. **10**, 143-153 [1990]). IFN- $\gamma$  has also been proposed for the treatment of atopic dermatitis, a common inflammatory skin disease characterized by severe pruritus, a chronically relapsing course with frequent periods of exacerbation, a distinctive clinical morphology and distribution of skin lesions (see PCT Publication No. WO 91/07984 published 13 June 1991), vascular stenosis, including the treatment of restenosis following angioplasty and/or vascular surgery (PCT Publication No. WO 90/03189 published 5 April 1990), various lung conditions, including respiratory distress syndromes (RDS), such as adult respiratory distress syndrome (ARDS) and a neonatal form, termed variously as idiopathic RDS or hyaline membrane disease (PCT Publication No. WO 89/01341, published 23 February 1989). In addition, IFN- $\gamma$  has been proposed for use in the treatment of various allergies, e.g. asthma, and HIV-infection-related conditions, such as opportunistic infections, e.g. *Pneumocystis carinii* pneumonia, and trauma-associated sepsis. Impaired IFN- $\gamma$  production has been observed in multiple-sclerosis (MP) patients, and it has been reported that the production of IFN- $\gamma$  is greatly suppressed in suspensions of mitogen-stimulated mononuclear cells derived from AIDS patients. For a review see, for example, Chapter 16, "The Presence of Possible Pathogenic Role of Interferons in Disease", In: Interferons and other Regulatory Cytokines, Edward de Maeyer (1988, John Wiley and Sons Publishers).

Interferon- $\gamma$ , along with other cytokines, has been implicated as an inducer of inducible nitric oxide (iNOS) which, in turn, has been described as an important mediator of the inflammatory mechanism underlying heart failure,

of the cardiac response to sepsis or allograft rejection, as well as the progression of dilated cardiomyopathies of diverse etiologies. Ungureanu-Longrois *et al.*, Circ. Res. **77**, 494-502 (1995); Pinsky *et al.*, J. Clin. Invest. **95**, 677-685 (1995); Singh *et al.*, J. Biol. Chem. **270**, 28471-8 (1995); Birks and Yacoub, Coronary Artery Disease **8**, 389-402 (1997); Hattori *et al.*, J. Mol. Cell. Cardiol. **29**, 1585-92 (1997). Indeed, IFN- $\gamma$  has been reported to be the most potent single cytokine with regard to myocyte iNOS induction (Watkins *et al.*, J. Mol. & Cell. Cardiol. **27**, 2015-29 [1995]).

### **Cardiac Hypertrophy**

Hypertrophy is generally defined as an increase in size of an organ or structure independent of natural growth that does not involve tumor formation. Hypertrophy of an organ or tissue is due either to an increase in the mass of the individual cells (true hypertrophy), or to an increase in the number of cells making up the tissue (hyperplasia), or both.

Cardiac hypertrophy is the enlargement of heart that is activated by both mechanical and hormonal stimuli and enables the heart to adapt to demands for increased cardiac output or to injury. Morgan and Baker, Circulation **83**, 13-25 (1991). This response is frequently associated with a variety of distinct pathological conditions, such as hypertension, aortic stenosis, myocardial infarction, cardiomyopathy, valvular regurgitation, cardiac shunt, congestive heart failure, etc.

On a cellular level, the heart functions as a syncytium of myocytes and surrounding support cells, called non-myocytes. While non-myocytes are primarily fibroblast/mesenchymal cells, they also include endothelial and smooth muscle cells. Indeed, although myocytes make up most of the adult myocardial mass, they represent only about 30% of the total cell numbers present in heart.

The enlargement of embryonic heart is largely dependent on an increase in myocyte number, which continues until shortly after birth, when cardiac myocytes lose their proliferative capacity. Further growth occurs through hypertrophy of the individual cells. Hypertrophy of adult cardiac ventricular myocytes is a response to a variety of conditions which lead to chronic hemodynamic overload. Thus, in response to hormonal, physiological, hemodynamic, and pathological stimuli, adult ventricular muscle cells can adapt to increased workloads through the activation of a hypertrophic process. This response is characterized by an increase in myocyte cell size and contractile protein content of individual cardiac muscle cells, without concomitant cell division and activation of embryonic genes, including the gene for atrial natriuretic peptide (ANP). Chien *et al.*, FASEB J. **5**, 3037-3046 (1991); Chien *et al.*, Annu. Rev. Physiol. **55**, 77-95 (1993). An increment in myocardial mass as a result of an increase in myocyte size that is associated with an accumulation of interstitial collagen within the extracellular matrix and around intramyocardial coronary arteries has been described in left ventricular hypertrophy secondary to pressure overload in humans (Caspari *et al.*, Cardiovasc. Res. **11**, 554-8 [1977]; Schwarz *et al.*, Am. J. Cardiol. **42**, 895-903 [1978]; Hess *et al.*, Circulation **63**, 360-371 [1981];

Pearlman *et al.*, Lab. Invest. **46**, 158-164 [1982]). Cardiac hypertrophy due to chronic hemodynamic overload is the common end result of most cardiac disorders and a consistent feature of cardiac failure.

It has also been suggested that paracrine factors produced by non-myocyte supporting cells may additionally be involved in the development of cardiac hypertrophy, and various non-myocyte derived hypertrophic factors, such as, leukocyte inhibitory factor (LIF) and endothelin, have been identified. Metcalf, Growth Factors **7**, 169-173 (1992); Kurzrock *et al.*, Endocrine Reviews **12**, 208-217 (1991); Inoue *et al.*, Proc. Natl. Acad. Sci. USA **86**: 2863-2867 (1989); Yanagisawa and Masaki, Trends Pharm. Sci. **10**, 374-378 (1989); U.S. Patent No. 5,573,762 (issued November 12, 1996). Further exemplary factors that have been identified as potential mediators of cardiac hypertrophy include cardiotrophin-1 (CT-1) (Pennica *et al.*, Proc. Nat. Acad. Sci. USA **92**:1142-1146 [1995]), catecholamines, adrenocorticosteroids, angiotensin, and prostaglandins.

Adult myocyte hypertrophy is initially beneficial as a short term response to impaired cardiac function by permitting a decrease in the load on individual muscle fibers. With severe, long-standing overload, however, the hypertrophied cells begin to deteriorate and die. Katz, "Heart Failure", in: Katz A.M. ed., Physiology of the Heart (New York, Raven Press, 1992) pp. 638-668. Cardiac hypertrophy is a significant risk factor for both mortality and morbidity in the clinical course of heart failure. Katz, Trends Cardiovasc. Med. **5**, 37-44 (1995).

For further details of the causes and pathology of cardiac hypertrophy see, e.g. Heart Disease, A Textbook of Cardiovascular Medicine, Braunwald, E. ed., W.B. Saunders Co., 1988, Chapter 14, Pathophysiology of Heart Failure.

### Treatment of cardiac hypertrophy

At present, the treatment of cardiac hypertrophy varies depending on the underlying cardiac disease. Catecholamines, adrenocorticosteroids, angiotensin, prostaglandins, leukemia inhibitory factor (LIF), endothelin (including endothelin-1, -2, and -3 and big endothelin), cardiotrophin-1 (CT-1) and cardiac hypertrophy factor (CHF) are among the factors identified as potential mediators of hypertrophy.

For example,  $\beta$ -adrenergic receptor blocking drugs ( $\beta$ -blockers, e.g., propranolol, timolol, tertalolol, carteolol, nadolol, betaxolol, penbutolol, acetobutolol, atenolol, metoprolol, carvedilol, etc.) and verapamil have been used extensively in the treatment of hypertrophic cardiomyopathy. The beneficial effects of  $\beta$ -blockers on symptoms (e.g. chest pain) and exercise tolerance are largely due to a decrease in the heart rate with a consequent prolongation of diastole and increased passive ventricular filling. Thompson *et al.*, Br. Heart J. **44**, 488-98 (1980); Harrison *et al.*, Circulation **29**, 84-98 (1964). Verapamil has been described to improve ventricular filling and probably reducing myocardial ischemia. Bonow *et al.*, Circulation **72**, 853-64 (1985). Nifedipine and diltiazem have also been used occasionally in the treatment of hypertrophic cardiomyopathy. Lorell *et al.*, Circulation **65**, 499-507 (1982); Betocchi *et al.*, Am. J. Cardiol. **78**, 451-7 (1996). However, because of its potent vasodilating properties, nifedipine may be

harmful, especially in patients with outflow obstruction. Disopyramide has been used to relieve symptoms by virtue of its negative inotropic properties. Pollick, *N. Engl. J. Med.* 307, 997-9 (1982). In many patients, however, the initial benefits decrease with time. Wigle *et al.*, *Circulation* 92, 1680-92 (1995).

Antihypertensive drug therapy has been reported to have beneficial effects on cardiac hypertrophy associated with elevated blood pressure. Examples of drugs used in antihypertensive therapy, alone or in combination, are calcium antagonists, e.g. nitrendipine;  $\beta$ -adrenergic receptor blocking agents, e.g., those listed above; angiotensin converting enzyme (ACE) inhibitors, e.g., quinapril, captopril, enalapril, ramipril, benazepril, fosinopril, lisinopril; diuretics, e.g. chlothiazide, hydrochlorothiazide, hydroflumethazide, methylchlothiazide, benzthiazide, dichlorphenamide, acetazolamide, indapamide; calcium channel blockers, e.g. diltiazem, nifedipine, verapamil, nicardipine. For example, treatment of hypertension with diltiazem and captopril showed a decrease in left ventricular muscle mass, but the Doppler indices of diastolic function did not normalize. Szlachcic *et al.*, *Am. J. Cardiol.* 63, 198-201 (1989); Shahi *et al.*, *Lancet* 336, 458-61 (1990). These findings were interpreted to indicate that excessive amounts of interstitial collagen may remain after regression of left ventricular hypertrophy. Rossi *et al.*, *Am. Heart J.* 124, 700-709 (1992). Rossi *et al.*, *supra*, investigated the effect of captopril on the prevention and regression of myocardial cell hypertrophy and interstitial fibrosis in pressure overload cardiac hypertrophy, in experimental rats.

As there is no generally applicable therapy for the treatment of cardiac hypertrophy, the identification of factors that can prevent or reduce cardiac myocyte hypertrophy is of primary importance in the development of new therapeutic strategies to inhibit pathophysiological cardiac growth.

## SUMMARY OF THE INVENTION

We have unexpectedly found that IFN- $\gamma$  inhibits the prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ )- and phenylephrine-induced spreading of cardiac myocytes isolated from adult rats. We have further found that IFN- $\gamma$  inhibits *in vivo* both cardiac hypertrophy induced by fluprostenol, an agonist analog of  $PGF_{2\alpha}$ , and hypertrophy induced by pressure overload in a rat model.

Accordingly, the present invention concerns the treatment of cardiac hypertrophy, regardless of the underlying cause, by administering a therapeutically effective dose of IFN- $\gamma$ . If the objective is the treatment of human patients, IFN- $\gamma$  preferably is recombinant human IFN- $\gamma$  (rhIFN- $\gamma$ ), most preferably, rhIFN- $\gamma$ -1b, which will be defined hereinbelow. The concept of treatment is used in the broadest sense, and specifically includes the prevention (prophylaxis), moderation, reduction, and curing of cardiac hypertrophy of any stage.

IFN- $\gamma$  preferably is administered in the form of a liquid pharmaceutical formulation, which may be preserved to achieve extended storage stability. Preserved liquid pharmaceutical formulations might contain multiple doses of IFN- $\gamma$ , and might, therefore, be suitable for repeated use.

IFN- $\gamma$  might be administered in combination with one or more further therapeutic agent used for the treatment of cardiac hypertrophy, or a physiological condition instrumental in the development of cardiac hypertrophy, such as elevated blood pressure, aortic stenosis, or myocardial infarction.

The invention further concerns a method for making a pharmaceutical composition for the treatment of cardiac hypertrophy, which comprises IFN- $\gamma$  as an active ingredient.

The invention also concerns a pharmaceutical product which comprises:

- (a) a pharmaceutical composition comprising at least one therapeutically effective dosage of IFN- $\gamma$ ;
- (b) a container containing said pharmaceutical composition; and
- (c) a label affixed to said container, or a package insert included in said pharmaceutical product referring to the use of said IFN- $\gamma$  in the treatment of cardiac hypertrophy.

### **BRIEF DESCRIPTION OF THE FIGURES**

In the Figures and throughout the examples, "IFN" or "IFN- $\gamma$ " refers to recombinant mouse IFN- $\gamma$  (Genentech, Inc., South San Francisco, CA, or Genzyme, Cambridge, MA).

**Figure 1** Inhibition of prostaglandin  $F_{2\alpha}$  ( $PGF_{2\alpha}$ )-induced spreading response by IFN- $\gamma$ . Myocytes were pre-incubated with saline vehicle or IFN- $\gamma$  (500 U/ml) on day of isolation. A second addition of vehicle or IFN- $\gamma$  was performed 24h after isolation, along with the addition of either vehicle or  $PGF_{2\alpha}$  ( $10^{-7}$  M). After an additional 72 hr incubation, cells were fixed in glutaraldehyde, stained with eosin Y and viewed by fluorescence microscopy. **A, B, C** Cardiac myocytes after 4 days in culture: control,  $PGF_{2\alpha}$ , and  $PGF_{2\alpha}$ +IFN- $\gamma$ , respectively. Histograms showing maximum breadth of rod shaped cardiac myocytes versus percent frequency of breadth occurrence. The maximum breadth of rod-shaped cells was determined by fluorescence microscopy and imaging software. At least 200 rod shaped cells from a single experiment were examined per group. IFN- $\gamma$  alone had no observable effect on the morphology of the cells.  $P < 0.001$  for all group comparisons.

**Figure 2** Dose responsive inhibition of  $PGF_{2\alpha}$ -induced response by IFN- $\gamma$  (500-25 U/ml). Myocytes were pre-incubated with saline vehicle or IFN- $\gamma$  on day of isolation. A second amount of IFN- $\gamma$  was added 24 hr after isolation, along with the addition of either vehicle or  $PGF_{2\alpha}$  ( $10^{-7}$  M). After an additional 72 hr incubation, cells were

fixed in glutaraldehyde, stained with eosin Y and viewed under fluorescence. Quantitation of myocyte morphology: **A** control, **B**  $\text{PGF}_{2\alpha}$ , **C**  $\text{PGF}_{2\alpha} + \text{IFN-}\gamma$  (25 U/ml), **D**  $\text{PGF}_{2\alpha} + \text{IFN-}\gamma$  (100 U/ml), **E**  $\text{PGF}_{2\alpha} + \text{IFN-}\gamma$  (500 U/ml). Histograms showing maximum breadth of rod shaped cardiac myocytes versus percent frequency of breadth occurrence. The maximum breadth of rod-shaped cells was determined by fluorescence microscopy and imaging software. At least 200 rod shaped cells from a single experiment were examined per group.  $\text{IFN-}\gamma$  alone had no observable effect on the morphology of the cells.  $P < 0.001$  for all group comparisons.

**Figure 3** Inhibition of phenylephedrine (PE)-induced spreading response by  $\text{IFN-}\gamma$ . Myocytes were pre-incubated with saline vehicle or  $\text{IFN-}\gamma$  (500 U/ml) on day of isolation. A second addition of vehicle or  $\text{IFN-}\gamma$  was performed 24h after isolation, along with the addition of either vehicle or PE ( $10^{-5}$  M). After an additional 72 hr incubation, cells were fixed in glutaraldehyde, stained with eosin Y and viewed by fluorescence microscopy. **A, B, C** Cardiac myocytes after 4 days in culture: control, PE, and PE+ $\text{IFN-}\gamma$ , respectively. Histograms showing maximum breadth of rod shaped cardiac myocytes versus percent frequency of breadth occurrence. The maximum breadth of rod-shaped cells was determined by fluorescence microscopy and imaging software. At least 200 rod shaped cells from a single experiment were examined per group.  $\text{IFN-}\gamma$  alone had no observable effect on the morphology of the cells.  $P < 0.001$  for all group comparisons.

**Figure 4** Effects of  $\text{IFN-}\gamma$  on cardiac hypertrophy induced by fluprostenol in rats. Data are presented as mean  $\pm$  SEM. The number in parentheses is the number of animals in each group. \*  $P < 0.05$ , \* $P < 0.01$ , compared to the vehicle group. # $P < 0.05$ , ## $P < 0.01$ , compared to the Flup group. Flup: fluprostenol;  $\text{IFN}$ = $\text{IFN-}\gamma$ ; HW: heart weight; BW: body weight; VW: ventricular weight; LVW: left ventricular weight.

**Figure 5** Effects of Flup and/or  $\text{IFN}$  on MAP and HR. Data are presented as mean  $\pm$  SEM. The number in parenthesis is the number of animals in each group. \*  $P < 0.05$ , compared to the vehicle group. #  $P < 0.05$ , compared to the Flup group. +  $P < 0.05$ , compared to the Flup+ $\text{IFN}$  group. Flup: fluprostenol;  $\text{IFN}$ :  $\text{IFN-}\gamma$ ; MAP: mean arterial pressure; HR: heart rate.

**Figure 6** Bargraphs showing the effect of fluprostenol (FLUP) and  $\text{IFN-}\gamma$  on: **A** Skeletal actin (SKA); **B** Sarcoplasmic reticulum calcium ATPase (SRCA); **C** Collagen I (COL I); **D** Atrial natriuretic factor (ANF) expression. Expression levels are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message. VEH is vehicle. There were 7 animals per group and the data are presented as the mean  $\pm$  SEM.  $P < 0.05$  vs VEH group.

**Figure 7** Effects of IFN- $\gamma$  on heart weight, ventricular weight, and left ventricular weight in rats with pressure overload. Data are presented as mean  $\pm$  SEM. The number in parenthesis is the number of animals in each group. \*\* P<0.01, compared to the sham group. ## P<0.01, compared to the Banded+vehicle group. Sham: sham-operated rats; Banded: aortic-banded rats; IFN: IFN- $\gamma$ ; HW: heart weight; VW: ventricular weight; LVW: left ventricular weight.

**Figure 8** Effects of IFN- $\gamma$  on the ratio of heart weight, ventricular weight, and left ventricular weight to body weight in rats with pressure overload. Data are presented as mean  $\pm$  SEM. The number in parenthesis is the number of animals in each group. \*\*P<0.01, compared to the sham group. ##P<0.01, compared to the Banded+vehicle group. Sham: sham-operated rats; Banded: aortic-banded rats; IFN: IFN- $\gamma$ ; HW: heart weight; BW: body weight; VW: ventricular weight; LVW: left ventricular weight.

**Figure 9** Effects of IFN- $\gamma$  on systolic arterial pressure, mean arterial pressure, and diastolic arterial pressure in rats with pressure overload. The number in parenthesis is the number of animals in each group. \*\*P<0.01, compared to the sham group. Sham: sham-operated rats; Banded: aortic-banded rats; IFN: IFN- $\gamma$ , SAP: systolic arterial pressure; MAP: mean arterial pressure; DAP: diastolic arterial pressure.

## DETAILED DESCRIPTION OF THE INVENTION

### **A. Definitions**

"Gamma interferon", "interferon-gamma", or "IFN- $\gamma$ " refers variously to all forms of (human and non-human animal) gamma interferon that are shown to be biologically active in any assay of cardiac hypertrophy, e.g. the hypertrophy assays disclosed herein, and is meant to include, but is not limited to, mature, pro, met and/or des(1-3) (also referred to as desCysTyrCys IFN- $\gamma$ ) form, whether obtained from natural source, chemically synthesized or produced by techniques of recombinant DNA technology. A complete description of the preparation of recombinant human IFN- $\gamma$  (rhuIFN- $\gamma$ ) including its cDNA and amino acid sequences are disclosed, for example, in United States Patent Nos. 4,727,138; 4,762,791; 4,925,793; 4,929,554; 5,582,824; 5,096,705; 4,855,238; 5,574,137; and 5,595,888. CysTyrCys-lacking recombinant human IFN- $\gamma$ , including variously truncated derivatives are, for example, disclosed in European Patent Publication No. 146,354. Non-human animal interferons, including IFN- $\gamma$ , are, for example, disclosed in European Publication No. 88,622. The term includes variously glycosylated forms and other variants (e.g. amino acid sequence variants) and derivatives of such native (wild-type) interferons, whether known in the art or will become available in the future. Examples of such variants are alleles, and the products of site-directed mutagenesis in which residues are deleted, inserted and/or substituted (see, e.g. European Publication No. 146,354 referred to above). IFN- $\gamma$



is known to have a narrow host range, therefore, IFN- $\gamma$  homologous to the animal to be treated should be used. In human therapy, the desCysTyrCys variant of the sequence shown in U. S. Patent No. 4,717,138 and its counterpart. EP 77,670, is preferably employed, and optionally the C-terminal variant in which the last four amino acid residues are deleted in post-translational processing. For human therapeutic use, the IFN- $\gamma$  of the present invention preferably is recombinant human IFN- $\gamma$  (rhIFN- $\gamma$ ), with or without the amino acids CysTyrCys at the N-terminus. More preferably, IFN- $\gamma$  is a recombinant human IFN- $\gamma$  species (recombinant human interferon gamma-1b, rhIFN- $\gamma$ -1b, containing 140 amino acids), which is the active ingredient of the commercial formulation, Actimmune® (Genentech, Inc., South San Francisco, California). As IFN- $\gamma$  is known to be highly species specific, in animal experiments, or for veterinary use. IFN- $\gamma$  of the animal species to be treated is preferably employed. Thus, in the *in vivo* experiments using a rat animal model, murine (mouse) recombinant IFN- $\gamma$  (Genentech, Inc.) has been employed. Rat and mice and sufficiently closely related to permit the use of mouse IFN- $\gamma$  in a rat model.

In a pharmacological sense, in the context of the present invention, a "therapeutically effective amount" of IFN- $\gamma$  refers to an amount effective in the treatment of hypertrophy, specifically cardiac hypertrophy.

"Hypertrophy", as used herein, is defined as an increase in mass of an organ or structure independent of natural growth that does not involve tumor formation. Hypertrophy of an organ or tissue is due either to an increase in the mass of the individual cells (true hypertrophy), or to an increase in the number of cells making up the tissue (hyperplasia), or both. Certain organs, such as the heart, lose the ability to divide shortly after birth. Accordingly, "cardiac hypertrophy" is defined as an increase in mass of the heart, which, in adults, is characterized by an increase in myocyte cell size and contractile protein content without concomitant cell division. The character of the stress responsible for inciting the hypertrophy, (e.g., increased preload, increased afterload, loss of myocytes, as in myocardial infarction, or primary depression of contractility), appears to play a critical role in determining the nature of the response. The early stage of cardiac hypertrophy is usually characterized morphologically by increases in the size of myofibrils and mitochondria, as well as enlargement of mitochondria and nuclei. At this stage, while muscle cells are larger than normal, cellular organization is largely preserved. At a more advanced stage of cardiac hypertrophy, there are preferential increases in the size or number of specific organelles, such a mitochondria, and new contractile elements are added in localized areas of the cells, in an irregular manner. Cells subjected to long-standing hypertrophy show more obvious disruptions in cellular organization, including markedly enlarged nuclei with highly lobulated membranes, which displace adjacent myofibrils and cause breakdown of normal Z-band registration. The phrase "cardiac hypertrophy" is used to include all stages of the progression of this condition, characterized by various degrees of structural damage of the heart muscle, regardless of the underlying cardiac disorder.

"Heart failure" refers to an abnormality of cardiac function where the heart does not pump blood at the rate needed for the requirements of metabolizing tissues. The heart failure can be caused by a number of factors, including ischemic, congenital, rheumatic, or idiopathic forms.

"Congestive heart failure" is a progressive pathologic state where the heart is increasingly unable to supply adequate cardiac output (the volume of blood pumped by the heart over time) to deliver the oxygenated blood to peripheral tissues. As congestive heart failure progresses, structural and hemodynamic damages occur. While these damages have a variety of manifestations, one characteristic symptom is ventricular hypertrophy. Congestive heart failure is a common end result of a number of various cardiac disorders.

"Myocardial infarction" generally results from atherosclerosis of the coronary arteries, often with superimposed coronary thrombosis. It may be divided into two major types: transmural infarcts, in which myocardial necrosis involves the full thickness of the ventricular wall, and subendocardial (nontransmural) infarcts, in which the necrosis involves the subendocardium, the intramural myocardium, or both, without extending all the way through the ventricular wall to the epicardium. Myocardial infarction is known to cause both a change in hemodynamic effects and an alteration in structure in the damaged and healthy zones of the heart. Thus, for example, myocardial infarction reduces the maximum cardiac output and the stroke volume of the heart. Also associated with myocardial infarction is a stimulation of the DNA synthesis occurring in the interstice as well as an increase in the formation of collagen in the areas of the heart not affected.

As a result of the increased stress or strain placed on the heart in prolonged hypertension due, for example, to the increased total peripheral resistance, cardiac hypertrophy has long been associated with "hypertension". A characteristic of the ventricle that becomes hypertrophic as a result of chronic pressure overload is an impaired diastolic performance. Fouad *et al.*, J. Am. Coll. Cardiol. 4, 1500-6 (1984); Smith *et al.*, J. Am. Coll. Cardiol. 5, 869-74 (1985). A prolonged left ventricular relaxation has been detected in early essential hypertension, in spite of normal or supranormal systolic function. Hartford *et al.*, Hypertension 6, 329-338 (1984). However, there is no close parallelism between blood pressure levels and cardiac hypertrophy. Although improvement in left ventricular function in response to antihypertensive therapy has been reported in humans, patients variously treated with a diuretic (hydrochlorothiazide), a  $\beta$ -blocker (propranolol), or a calcium channel blocker (diltiazem), have shown reversal of left ventricular mass, without improvement in diastolic function. Inouye *et al.*, Am. J. Cardiol. 53, 1583-7 (1984).

Another complex cardiac disease associated with cardiac hypertrophy is "hypertrophic cardiomyopathy". This condition is characterized by a great diversity of morphologic, functional, and clinical features (Maron *et al.*, N. Engl. J. Med. 316, 780-9 [1987]; Spirito *et al.*, N. Engl. J. Med. 320, 749-55 [1989]; Louie and Edwards, Prog. Cardiovasc. Dis. 36, 275-308 [1994]; Wagle *et al.*, Circulation 92, 1680-92 [1995]), the heterogeneity of which is accentuated by the fact that it afflicts patients of all ages (Spirito *et al.*, N. Engl. J. Med. 336, 775-785 [1997]). The causative factors

of hypertrophic cardiomyopathy are also diverse and little understood. Recent data suggest that  $\beta$ -myosin heavy chain mutations may account for approximately 30 to 40 percent of cases of familial hypertrophic cardiomyopathy (Watkins *et al.*, N. Engl. J. Med. **326**, 1108-14 [1992] Schwartz *et al.*, Circulation **91**, 532-40 [1995]; Marian and Roberts, Circulation **92**, 1336-47 [1995]; Thierfelder *et al.*, Cell **77**, 701-12 [1994]; Watkins *et al.*, Nat. Gen. **11**, 434-7 [1995]).

5       Supravalvular "aortic stenosis" is an inherited vascular disorder, that is characterized by narrowing of the ascending aorta, but other arteries, including the pulmonary arteries, may also be affected. Untreated aortic stenosis may lead to increased intracardiac pressure resulting in myocardial hypertrophy and eventually heart failure and death. The pathogenesis of this disorder is not fully understood, but hypertrophy and possibly hyperplasia of medial smooth muscle are prominent features of this disorder. It has been reported that molecular variants of the elastin gene are involved in  
10       the development and pathogenesis of aortic stenosis. (U.S. Patent No. 5,650,282 issued July 22, 1997.)

      "Valvular regurgitation" occurs as a result of heart diseases resulting in disorders of the cardiac valves. Various diseases, like rheumatic fever, can cause the shrinking or pulling apart of the valve orifice, while other diseases may result in endocarditis, an inflammation of the endocardium or lining membrane of the atrioventricular orifices and operation of the heart. Defects such as the narrowing of the valve stenosis or the defective closing of the valve result  
15       in an accumulation of blood in the heart cavity or regurgitation of blood past the valve. If uncorrected, prolonged valvular stenosis or insufficiency may result in cardiac hypertrophy and associated damage to the heart muscle, which may eventually necessitate valve replacement.

      The treatment of all these, and other cardiac disorders accompanied by cardiac hypertrophy is subject of the present invention.

20       "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) hypertrophy. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented. The hypertrophy may result from any cause, including idiopathic, cardiotrophic, or myotrophic causes, or ischemia or ischemic insults, such as myocardial infarction.

25       "Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial antihypertrophic effect for an extended period of time.

      "Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cows, horses, sheep, pigs, etc. Preferably, the mammal is human.

30       Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

**B. Modes of Carrying out the Invention****1. Cardiac hypertrophy assays***In vitro assays***a. Induction of spreading of adult rat cardiac myocytes**

In this assay, ventricular myocytes are isolated from a single (male Sprague-Dawley) rat, essentially following a modification of the procedure described in detail by Piper *et al.*, "Adult ventricular rat heart muscle cells." In: Cell Culture Techniques in Heart and Vessel Research, H.M. Piper, ed., Berlin: Springer-Verlag, 1990, pp. 36-60. This procedure permits the isolation of adult ventricular myocytes and the long-term culture of these cells in the rod-shaped phenotype. Phenylephrine and Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) have been shown to induce a spreading response in these adult cells. Piper *et al.*, *supra*; Lai *et al.*, Am J. Physiol. 1996; 271 (Heart Circ. Physiol. 40):H2197-H2208. The inhibition of myocyte spreading induced by PGF<sub>2α</sub>, or PGF<sub>2α</sub> analogs, (e.g. fluprostenol) and phenylephrine by various potential inhibitors of cardiac hypertrophy is then tested. A detailed protocol is described in the examples that follow.

*In vivo assays***a. Inhibition of cardiac hypertrophy induced by fluprostenol *in vivo***

This pharmacological model tests the ability of IFN-γ to inhibit cardiac hypertrophy induced in rats (e.g. male Wistar or Sprague-Dawley) by subcutaneous injection of fluprostenol (an agonist analog of PGF<sub>2α</sub>). It is known that rats with pathologic cardiac hypertrophy induced by myocardial infarction have chronically elevated levels of extractable PGF<sub>2α</sub> in their myocardium. Lai *et al.*, Am. J. Physiol. (Heart Circ. Physiol.) 271:H2197-H2208 (1996). Accordingly, factors that can inhibit the effects of fluprostenol on myocardial growth *in vivo* are potentially useful for treating cardiac hypertrophy. The effects of IFN-γ on cardiac hypertrophy are determined by measuring the weight of heart, ventricles, and left ventricle (normalized by bodyweight) relative to fluprostenol-treated rats not receiving IFN-γ. A detailed description of this assay is provided in the examples.

**b. Pressure-overload cardiac hypertrophy assay.**

For *in vivo* testing it is common to induce pressure-overload cardiac hypertrophy by constriction of the abdominal aorta of test animals. In a typical protocol rats (e.g. male Wistar or Sprague-Dawley) are treated under anesthesia, and the abdominal aorta of each rat is narrowed down just below the diaphragm. Beznak M., Can. J. Biochem. Physiol. 33, 985-94 (1955). The aorta is exposed through a surgical incision, and a blunted needle is placed next to the vessel. The aorta is constricted with a ligature of wool thread around the needle, which is immediately removed and which reduces the lumen of the aorta to the diameter of the needle. This approach is described, for example, in Rossi *et al.*, Am. Heart J. 124, 700-709 (1992) and O'Rourke and Reibel, P.S.E.M.B. 200, 95-100 (1992). A detailed description of the protocol used by the present inventors is disclosed in the examples hereinbelow.

b. Effect on cardiac hypertrophy following experimentally induced myocardial infarction (MI).

Acute MI is induced in rats by left coronary artery ligation and confirmed by electrocardiographic examination. A sham-operated group of animals is also prepared as control animals. Earlier data have shown that cardiac hypertrophy is present in the group of animals with MI, as evidenced by an 18% increase in heart weight-to-body weight ratio. Lai *et al., supra*. Treatment of these animals with candidate blockers of cardiac hypertrophy, e.g. IFN- $\gamma$  provides valuable information about the therapeutic potential of the candidates tested.

2. Uses, therapeutic compositions and administration of IFN- $\gamma$

In accordance with the present invention, IFN- $\gamma$  can be used for the treatment of cardiac hypertrophy, i.e. the enlargement of heart, regardless of the etiology and pathogenesis. When an excessive pressure or volume load is imposed on the heart (ventricle), cardiac (myocardial) hypertrophy develops, providing a fundamental compensatory mechanism that permits the ventricle to sustain its burden. Krayenbuehl *et al.*, Eur. Heart J. **4** (Suppl. A), 29 (1983). The character of the stress (increased preload, increased afterload, loss of myocytes, as in myocardial infarction, or primary depression of contractility) responsible of the development of hypertrophy plays a critical role in determining the nature of the hypertrophic response. Scheuer and Buttrick, Circulation **75**(Suppl. I), 63 (1987). The present invention concerns the treatment of cardiac hypertrophy associated with any underlying pathological condition, including, without limitation, post myocardial infarction, hypertension, aortic stenosis, cardiomyopathy, valvular regurgitation, cardiac shunt, and congestive heart failure. The main characteristics of these conditions have been discussed hereinabove.

Particularly important is the use of IFN- $\gamma$  for the prevention of cardiac failure following myocardial infarction. About 750,000 patients suffer from acute myocardial infarction (AMI) annually, and approximately one-fourth of all death in the United States are due to AMI. In recent years, thrombolytic agents, e.g. streptokinase, urokinase, and in particular tissue plasminogen activator (t-PA) have significantly increased the survival of patients who suffered myocardial infarction. When administered as a continuous intravenous infusion over 1.5 to 4 hours, t-PA produces coronary patency at 90 minutes in 69% to 90% of the treated patients. Topol *et al.*, Am. J. Cardiol. **61**, 723-8 (1988); Neuhaus *et al.*, J. Am. Coll. Cardiol. **12**, 581-7 (1988); Neuhaus *et al.*, J. Am. Coll. Cardiol. **14**, 1566-9 (1989). The highest patency rates have been reported with high dose or accelerated dosing regimens. Topol, J. Am. Coll. Cardiol. **15**, 922-4 (1990). t-PA may also be administered as a single bolus, although due to its relatively short half-life, it is better suited for infusion therapy. Tebbe *et al.*, Am. J. Cardiol. **64**, 448-53 (1989). A t-PA variant, specifically designed to have longer half-life and very high fibrin specificity, TNK t-PA (a T103N, N117Q, KHRR(296-299)AAAA t-PA variant, Keyt *et al.*, Proc. Natl. Acad. Sci. USA **91**, 3670-3674 (1994)) is particularly suitable for bolus administration. However, despite all these advances, the long-term prognosis of patient survival depends greatly on the

post-infarction monitoring and treatment of the patients, which should include monitoring and treatment of cardiac hypertrophy.

Another important therapeutic indication is the treatment of cardiac hypertrophy associated with hypertension. As noted before, sustained hypertension is known to result in cardiac hypertrophy. Although certain hypotensive agents have been shown to reduce left ventricular mass, treatment does not always result in the improvement of diastolic function. Accordingly, IFN- $\gamma$  can be administered in combination with  $\beta$ -adrenergic receptor blocking agents, e.g., propranolol, timolol, tertalolol, carteolol, nadolol, betaxolol, penbutolol, acetobutolol, atenolol, metoprolol, carvedilol; angiotensin converting enzyme (ACE) inhibitors, e.g., quinapril, captopril, enalapril, ramipril, benazepril, fosinopril, lisinopril; diuretics, e.g. chorothiazide, hydrochlorothiazide, hydroflumethazide, methylchlorothiazide, benzthiazide, dichlorphenamide, acetazolamide, indapamide; and/or calcium channel blockers, e.g., diltiazem, nifedipine, verapamil, nocardipine. Pharmaceutical compositions comprising the therapeutic agents identified here by their generic names are commercially available, and are to be administered following the manufacturers' instructions for dosage, administration, adverse effects, contraindications, etc. (See, e.g. Physicians' Desk Reference, Medical Economics Data Production Co., Montvale, N.J., 51th Edition, 1997.)

IFN- $\gamma$  may also be administered prophylactically to patients with cardiac hypertrophy, to prevent the progression of the condition, and avoid sudden death, including death of asymptomatic patients. Such preventative therapy is particularly warranted in the case of patients diagnosed with massive left ventricular cardiac hypertrophy (a maximal wall thickness of 35 mm or more in adults, or a comparable value in children), or in instances when the hemodynamic burden on the heart is particularly strong.

IFN- $\gamma$  may also be useful in the management of atrial fibrillation, which develops in a substantial portion of patients diagnosed with hypertrophic cardiomyopathy.

IFN- $\gamma$  is administered in the form of a pharmaceutical composition comprising IFN- $\gamma$  as an active ingredient, in conjunction with a pharmaceutically acceptable carrier. Therapeutic formulations of IFN- $\gamma$  for treating cardiac hypertrophy are prepared for storage by mixing IFN- $\gamma$  having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences, *supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients, or stabilizers are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics, or polyethylene glycol (PEG).

IFN- $\gamma$  to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. IFN- $\gamma$  ordinarily will be stored in lyophilized form or in solution.

IFN- $\gamma$  may be used in lyophilized form, in combination with other ingredients for reconstitution with an appropriate diluent at the time for use. Because IFN- $\gamma$  is known to be acid labile, it has traditionally been handled at neutral or slightly alkaline pH. See, for example, U.S. Patent No. 4,499,014 which discloses reactivation of a lyophilized acidic IFN- $\gamma$  solution to a pH of 6 to 9. Neutral or slightly alkaline solutions of higher concentrations of IFN- $\gamma$  are generally unsuitable as injectable formulations because of the immediate formation of a visible precipitate. Such precipitate may cause thrombosis on administration or decrease potency. European Patent Publication No. 0196,203 discloses reconstitution of lyophilized IFN- $\gamma$  to a pH of 4 to 6.0.

Stable liquid pharmaceutical compositions comprising an effective amount of non-lyophilized IFN- $\gamma$  along with a buffer capable of maintaining the pH at 4.0-6.0, a stabilizing agent, and a non-ionic detergent are disclosed in U.S. Patent No. 5,151,265 issued 29 September 1992. The stabilizing agent typically is a polyhydric sugar alcohol, such as mannitol, and the non-ionic detergent may be a surfactant, e.g. polysorbate 80 or polysorbate 20. The non-ionic detergent preferably is present in a range of about 0.07 to 0.2 mg/ml, and most preferably in a concentration of about 0.1 mg/ml. Suitable buffers are conventional buffers of organic acids and salt thereof, such as nitrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid-monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid-disodium succinate mixture, etc.), tartarate buffers (e.g., tartaric acid-sodium tartarate mixture, tartaric acid-potassium tartarate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g. fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g. gluconic acid-sodium gluconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.), oxalate buffers (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc.), lactate buffers (e.g. lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.), and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.)

A known commercial liquid formulation of IFN- $\gamma$  (Actimmune® rhuIFN- $\gamma$ -1b, Genentech, Inc.) is a sterile, clear, colorless unpreserved solution filled in a single-dose vial for subcutaneous injection. Each 0.5 ml vial of Actimmune contains 100  $\mu$ g (3 million U, specific activity: 30 million U/mg) of IFN- $\gamma$ -1b formulated in 20 mg mannitol, 0.36 mg sodium succinate, 0.05 mg polysorbate 20 and Sterile Water for Injection.

Preserved pharmaceutical compositions to be used in accordance with the present invention, which are suitable for repeated use, preferably contain:

- a) IFN- $\gamma$  not subjected to prior lyophilization;
- b) an acetate buffer capable of maintaining the pH between about 4 and about 6 (the pH range of maximum stability of the protein in solution);
- c) a non-ionic detergent primarily to stabilize the protein against agitation-induced aggregation;
- d) an isotonicifier;
- e) a preservative selected from the group of phenol, benzyl alcohol and a benzethonium halide, e.g. chloride; and
- f) water.

The non-ionic detergents (surfactants) may, for example, be polysorbates (e.g. polysorbate [Tween] 20, 80, etc.) or poloxamers (e.g. poloxamer 188). The use of non-ionic surfactants permits the formulation to be exposed to shear surface stresses without causing denaturation of the protein. Further, such surfactant containing formulations may be employed in aerosol devices such as those used in a pulmonary dosing, and needleless jet injector guns (see, e.g. EP 257,956).

The isotonicifier is present to ensure isotonicity of the liquid compositions of the present invention, and includes polyhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. These sugar alcohols can be used alone or in combination. Alternatively, sodium chloride or other appropriate inorganic salts may be used to render the solutions isotonic.

The acetate buffer may, for example, be an acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc. The pH of the liquid formulation of this invention is buffered in the range of about 4.0 to 6.0, preferably 4.5 to 5.5, and most preferably at about pH 5.

The preservatives phenol, benzyl alcohol and benzethonium halides, e.g. chloride, are known antimicrobial agents.

In a preferred embodiment, IFN- $\gamma$  is administered in the form of a liquid pharmaceutical composition which comprises the following components:

IFN- $\gamma$	0.1-2.0 mg/ml
sodium acetate (pH 5.0)	5-100 mM
Tween 20	0.1 to 0.01 % by weight
phenol	0.05 to 0.4 % by weight
mannitol	5 % by weight
water for injection, USP	up to 100 %,

wherein the percentage amounts are based on the weight of the composition. Phenol can be replaced by 0.5-1.0 % by weight of benzyl alcohol, and mannitol can be replaced by 0.9 % by weight sodium chloride.



Most preferably, the compositions comprise

IFN- $\gamma$	0.1 to 1.0 mg/ml
sodium acetate (pH 5.0)	10 mM
Tween 20	0.01 % by weight
phenol	0.2 %
mannitol	5 %

Phenol can be replaced by 0.75 % by weight benzyl alcohol and mannitol by 0.9 % by weight sodium chloride.

The preserved liquid formulations preferably contain multiple doses of a therapeutically effective amount of IFN- $\gamma$ . In view of the narrow host range of this polypeptide, for the treatment of human patients liquid formulations comprising human IFN- $\gamma$ , more preferably native sequence human IFN- $\gamma$ , are preferred. As a biological response modifier, IFN- $\gamma$  exerts a wide variety of activities on a wide range of cell types, in a variety of human and non-human mammalian species. The therapeutically effective dose will, of course, vary depending on such factors as the pathological condition to be treated (including prevention), the patient's age, weight, general medical condition, medical history, etc., and its determination is well within the skill of a practicing physician. The effective dose generally is within the range of from about 0.001 to about 1.0 mg/kg, more preferably about 0.01-1 mg/kg, most preferably about 0.01-0.1 mg/kg. In such formulations huIFN- $\gamma$  will preferably exhibit a specific activity of on the order of about  $2 \times 10^7$  U/mg of protein or greater when tested on A549 cells against encephalomyocarditis virus. It should be appreciated that endotoxin contamination should be kept minimally at a safe level, for example, less than 0.5 ng/mg protein. Moreover, for human administration, the liquid formulations should meet sterility, pyrogenicity, general safety, and purity as required by FDA Office and Biologics standards.

The route of IFN- $\gamma$  administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained-release systems as noted below. Therapeutic IFN- $\gamma$  compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The formulations are preferably administered as repeated intravenous (i.v.), subcutaneous (s.c.) or intramuscular (i.m.) injections, or as aerosol formulations suitable for intranasal or intrapulmonary delivery (for intrapulmonary delivery see, *e.g.* EP 257,956).

The stable aqueous compositions of IFN- $\gamma$  are preferably contained in vials, containing up to about 30 therapeutically effective doses of IFN- $\gamma$ . The bioactivity of IFN- $\gamma$  preferably remains within about 20 % from the bioactivity exhibited at the time of first administration for at least about 14 days, more preferably for at least about 200 days following first administration.

IFN- $\gamma$  can also be administered in the form of sustained-released preparations. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, J. Biomed. Mater. Res., 15: 167-277 [1981] and Langer, Chem. Tech., 12: 98-105 [1982] or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, Biopolymers, 22: 547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues. Lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release IFN- $\gamma$  compositions also include liposomally entrapped IFN- $\gamma$ . Liposomes containing IFN- $\gamma$  are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. USA, 82: 3688-3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. USA, 77: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Patent Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal therapy.

An effective amount of IFN- $\gamma$  to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. The recommended dosage for the administration of IFN- $\gamma$  (Actimmune®, Genentech, Inc.) to treat patients with chronic granulomatous disease is 50 mcg/m<sup>2</sup> (1.5 million U/m<sup>2</sup>) for patients whose body surface area is greater than 0.5 m<sup>2</sup>, and 1.5 mcg/kg/dose for patients whose body surface area is equal to or less than 0.5 m<sup>2</sup>, administered as a subcutaneous injection, three times a week. This is valuable guidance for a physician to determine the optimal effective dose for the treatment of cardiac hypertrophy. The clinician will administer IFN- $\gamma$  until a dosage is reached that achieves the desired effect for treatment of the heart dysfunction. For example, if the objective is the treatment of congestive heart

failure, the amount would be one which inhibits the progressive cardiac hypertrophy associated with this condition. The progress of this therapy is easily monitored by echo cardiography. Similarly, in patients with hypertrophic cardiomyopathy, IFN- $\gamma$  can be administered on an empirical basis, relying on the patient's subjective perception of benefit.

IFN- $\gamma$  may be administered in combination with other therapeutic agents used for the treatment (including prevention) of cardiac hypertrophy. For example, IFN- $\gamma$  therapy can be combined with the administration of inhibitors of known cardiac myocyte hypertrophy factors, e.g. inhibitors of  $\alpha$ -adrenergic agonists, e.g. phenylephrine; endothelin-1; CT-1; LIF; angiotensin converting enzyme; and angiotensin II. Inhibitors of cardiac hypertrophy factor (CHF, cardiotrophin or cardiotrophin-1, see, e.g. US 5,679,545) are particularly preferred for combination therapy.

Preferred candidates for combination therapy in the treatment of hypertrophic cardiomyopathy are  $\beta$ -adrenergic-blocking drugs (e.g., propranolol, timolol, tertalolol, carteolol, nadolol, betaxolol, penbutolol, acetobutolol, atenolol, metoprolol, carvedilol), verapamil, diltiazem. Treatment of hypertrophy associated with high blood pressure may require the use of antihypertensive drug therapy, using calcium channel blockers, e.g. diltiazem, nifedipine, verapamil, nicardipine;  $\beta$ -adrenergic blocking agents; diuretics, e.g., chlorothiazide, hydrochlorothiazide, hydroflumethazide, methylchlorothiazide, benzthiazide, dichlorphenamide, acetazolamide, indapamide; and/or ACE-inhibitors, e.g., quinapril, captopril, enalapril, ramipril, benazepril, fosinopril, lisinopril.

The effective amount of the therapeutic agents administered in combination with IFN- $\gamma$  will be at the physician's or veterinarian's discretion. Dosage administration and adjustment is done to achieve optimal management of the conditions to be treated, and ideally takes into account use of diuretics or digitalis, and conditions such as hyper- or hypotension, renal impairment, etc. The dose will additionally depend on such factors as the type of the therapeutic agent to be used and the specific patient being treated. Typically, the amount employed will be the same dose as that used, if the given therapeutic agent is administered without IFN- $\gamma$ .

## EXAMPLES

### **Example 1**

#### Inhibition of PGF<sub>2 $\alpha$</sub> -induced spreading response of adult myocytes by IFN- $\gamma$

##### Materials and Methods

**Adult myocyte cultures** The procedure used for the isolation of ventricular myocytes from adult rats was a modification of a procedure described by Piper *et al.*, *supra*, and is detailed in Lai *et al.*, *supra*. For each myocyte preparation, one male Sprague-Dawley rat weighing about 250 g was anesthetized with pentobarbital sodium, and the heart was removed. Extraneous tissue was trimmed from the heart, and it was mounted onto a Langendorff system that was temperature controlled at 37°C. The heart was perfused with about 40 ml of Krebs buffer (110 mM NaCl, 2.6 mM

KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 25 mM  $\text{NaHCO}_3$  and 11 mM glucose). A solution containing 30 mg of collagenase and 12.5  $\mu\text{l}$  of 100 mM  $\text{CaCl}_2$  in 50 ml of Krebs buffer was then recirculated through the heart for 30 minutes. The heart was removed from the Langendorff apparatus, and the atria and connective tissues were removed. The ventricles were cut into 2 mm cubes with dissecting scissors, and further digested in a fresh collagenase solution (30 mg collagenase and 400 mg BSA dissolved in Krebs buffer with 12.5  $\mu\text{l}$  of 100 mM  $\text{CaCl}_2$ ) for five minutes at 37 °C. During the digestion, the tissue suspension was gently hand shaken one time per minute. After the digestion, the supernatant was removed and saved, and the remaining tissue was further digested in fresh collagenase solution for an additional five minutes.

Isolated adult rat myocytes were plated on laminin-coated plates at a density of  $3 \times 10^3$  cells/ml. After 72 hours of appropriate stimulation, the cells were fixed with gluteraldehyde and stained with Eosin Y. Images of rod shaped cells were captured under fluorescent microscopy and maximum breadth was determined using imaging software (Simple 32, Compix Imaging, Mars, PA).

## Results

*IFN- $\gamma$  inhibits the spreading of adult cardiac myocyte induced by the hypertrophy factors  $\text{PGF}_{2\alpha}$  and phenylephrine*

$\text{PGF}_{2\alpha}$  and the  $\alpha$ -adrenergic agonist phenylephrine have been shown to induce hypertrophy of cultured neonatal rat cardiac myocytes (Adams *et al.*, J. Biol. Chem. 271:1179-1186 [1996]; Lai *et al.*, Am. J. Physiol. (Heart Circ. Physiol.) 271: H2197-H2208 [1996]; Meidell *et al.*, Am. J. Physiol. 251: H1076-H1084 [1986]; Simpson, J. Clin. Invest. 72:732-738 [1983]; Simpson, Circ. Res. 56:884-894 [1985]). Adult rat ventricular myocytes spread when exposed to these factors in culture (Lai *et al.*, *supra*; Piper *et al.*, "Adult ventricular rat heart muscle cells", in: Cell Culture Techniques in Heart and Vessel Research, H.M. Piper, Editor, 1990, Springer-Verlag: Berlin, p. 36-60.) Adult myocytes have a rod-like morphology. When these cells are exposed to 0.1  $\mu\text{M}$   $\text{PGF}_{2\alpha}$ , the rod shaped cells flatten and spread (Figure 1). The spreading response was quantified by measuring the maximum cell breadth of at least 200 rod shaped cells and plotting this value vs the percent frequency of cell breadth in the population.  $\text{PGF}_{2\alpha}$  induced a significant change in the maximum cell breadth as evidenced by a shift in the population distribution for this parameter compared to control cells ( $P < 0.001$ ). Treating cells with IFN- $\gamma$  significantly inhibited their response to  $\text{PGF}_{2\alpha}$  ( $P < 0.001$   $\text{PGF}_{2\alpha}$  + IFN- $\gamma$  compared to  $\text{PGF}_{2\alpha}$ ). The inhibitor effect of IFN- $\gamma$  on  $\text{PGF}_{2\alpha}$ -induced myocyte spreading was dose dependent (Figure 2) over a concentration range that is consistent with the biological response to IFN- $\gamma$  in cardiac myocytes and other cell systems (Singh *et al.*, J. Biol. Chem. 271: 1111-1117 [1996]; Pinsky *et al.*, J. Clin. Invest. 95:766-685 [1995]; Ungureanu-Longrois *et al.*, Circ. Res. 77: 494-502 [1995];

Soderberg-Naucleer *et al.*, *J. Clin. Invest.* 100:3154-3163 [1997]; Gou *et al.*, *J. Clin. Invest.* 100:829-838 [1997]; Marra *et al.*, *Can J. Cardiol.* 12:1259-1267 [1996]). The ability to inhibit PGF<sub>2α</sub> induced myocyte spreading appears to be specific to IFN-γ since several other cytokines including IL-1α, IL-1β, IL-2, IL-6, TNF-α, IFN-α, and IFN-β could not inhibit the spreading response. The inhibitory effect of IFN-γ is not specific for PGF<sub>2α</sub>. IFN-γ can also inhibit spreading induced by phenylephrine (Figure 3).

## Example 2

### Inhibition of cardiac hypertrophy *in vivo*

#### Materials and Methods

**Animals** All experimental procedures conformed to the guiding principles of the American Physiology Society, and were approved by Genentech's Institutional Animal Care and Use Committee. The animals used in this study were male Sprague/Dawley (SD) rats (8 weeks of age, Charles River Breeding Laboratories, Inc.). The animals were acclimated to the facility for at least one week before experiments, fed a pelleted rat chow and water *ad libitum*, and housed in a light and temperature controlled room.

**Administration of fluprostenol and/or IFN-γ** Rats received subcutaneous injection of fluprostenol (Cayman Chemical, Ann Arbor, MI) at 0.15 mg/kg, recombinant mouse IFN-γ (Genentech, Inc., South San Francisco, CA) at 0.08 mg/kg, combination of fluprostenol and IFN-γ, or saline vehicle, twice a day for 14 days. In the IFN-γ and fluprostenol+IFN-γ groups, animals were pretreated with IFN-γ for one day. Body weight was measured before and after treatment. A previous study has shown that the dose of fluprostenol used here is the lowest dose which produces a significant cardiac hypertrophy in rats. Lai *et al.*, *supra*. A pilot study demonstrated that IFN-γ at the dose indicated above inhibited fluprostenol-induced cardiac hypertrophy with little effects on body weight in rats.

**Hemodynamic assessment** Thirteen days after treatment, rats were anesthetized with intraperitoneal injection of ketamine 80 mg/kg (Aveco Co., Inc., Fort Dodge, Iowa) and xylazine 10 mg/kg (Rugby Laboratories, Inc., Rockville Center, NY.). A catheter (PE-10 fused with PE 50) filled with heparin-saline solution (50 U/ml) was implanted into the abdominal aorta, via the right femoral artery, for measurement of mean arterial pressure (MAP) and heart rate (HR). The catheter was exteriorized and fixed at the back of the neck.

One day after catheterization, the arterial catheter was connected to a Model CP10 pressure transducer (Century Technology Company, Inglewood, CA, USA) that was coupled to a Grass Model 7 polygraph (Grass Instruments, Quincy, MA, USA). MAP and HR were measured simultaneously in conscious, unrestrained rats.

**Measurement of organ weights** Under anesthesia with ketamine/xylazine, the heart, kidney, and spleen were removed, dissected and weighed. The left ventricle was stored at 80°C for evaluation of gene expression.

*Animal model of pressure overload*

The induction of pressure overload by partial ligation of the abdominal aorta in rats was as described previously. Kimura *et al.*, Am. J. Physiol. 1989:256 (Heart Circ. Physiol. 25):H1006-H1-11; Batra *et al.*, J. Cardiovasc. Pharmacol. 17(suppl. 2), S151-S153 (1991). In brief, rats were anesthetized with ketamine/xylazine as described above. A 3 cm midline incision was made in the abdominal wall. The abdominal aorta between the diaphragm and the renal artery was exposed and looped with 5-0 silk suture. The suture was tightened around a gauge 23 needle, and then the needle was withdrawn. Sham animals received the surgery without tightening the suture.

*Experimental protocol in rats with pressure overload*

The rats with aortic banding randomly received subcutaneous injection of IFN- $\gamma$  at 0.08 mg/kg twice a day for one day before surgery and for 14 days after surgery. Sham animals were not treated. Thirteen days after treatment, a catheter was implanted into the right carotid artery under anesthesia as indicated above. One day after implantation, arterial pressure and HR were measured in conscious rats. The heart and other organs including the liver, kidney, and spleen were removed, weighed, and fixed in 10% buffered formalin for pathological studies. The left ventricle was quickly dissected and frozen with liquid nitrogen in some animals and stored at -80°C for assessment of gene expression.

*Statistical analysis*

Results are expressed as mean $\pm$ SEM. One way analysis of variance (ANOVA) was performed to assess differences in parameters between groups. Significant differences were then subjected to *post hoc* analysis using Newman-Keuls method;  $P < 0.05$  was considered significant.

*RNA preparation* Total RNA was isolated using RNeasy Maxi Columns (Qiagen) according to the manufacturer's instructions.

*RT-PCR*

Real-time RT-PCR (TaqMan) technology was used to compare the gene expression between the various treatment groups. An oligonucleotide probe containing a fluorescent reporter dye, 6-carboxytetramethylrhodamine (TAMRA), at the 3'-end was designed to hybridize to the amplicon defined by two PCR primers. A 3'-blocking phosphate prevents extension of the probe. The reporter dye is released from the probe by the 5' exonuclease activity of Taq polymerase during the extension phase of PCR reaction. The resulting fluorescence is monitored in the reaction tube by the sequence detector and quantified without further manipulation, hence the term "real-time". The threshold cycle number (Ct), defined as the point where the reporter fluorescence reaches a value greater than 10 times the standard deviation of the baseline, is proportional to the amount of amplicon produced from the sample. Since the fluorescence is detected during the exponential phase of the amplification, none of the reaction components are limiting. In each experiment, a control is analyzed that lacks the RNA template to monitor for contamination, and another control is included where the RT step is omitted to eliminate amplification of possible contaminating DNA as the source of the signal. Reactions are optimized to give the greatest fluorescence signal and smallest Ct by titration of magnesium and primer concentrations, and the product is run on an agarose gel to verify the presence of a single band at the predicted

molecular weight. In addition, the sequence of the amplicon is screened against Genbank to eliminate the possibility of overlap with closely related genes.

For each sample the mRNA for each target gene is determined using a standard curve as described below and then normalized to the amount of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) in the sample (see below for specifics of this calculation). The relative abundance of each target gene to GAPDH can then be compared among treatment groups.

RT-PCR was performed on 1 ng of total RNA per reaction using the TaqMan Model 7700 Sequence Detector (ABI-Perkin Elmer) (Gibson *et al.*, Genome Res. 6, 995-1001 [1996]). Amplification reaction conditions (for 50  $\mu$ l) were 1x TaqMan Buffer A, 200  $\mu$ M dATP, dCTP, dGTP, and 400  $\mu$ M dUTP, 10% glycerol, 6.5 mM MgCl<sub>2</sub>, 50 U MuLV reverse transcriptase, 20 U RNase Inhibitor, 1.25 U AmpliTaq Gold, 100 nM forward and reverse primers, and 100 nM fluorogenic probe. RT-PCR reagents and glycerol were purchased from Perkin Elmer and Sigma, respectively. Reactions were performed in MicoAmp Optical Tubes and Caps (ABI-Perkin Elmer). TaqMan primers and probes were designed according to guidelines determined by Perkin Elmer and synthesized at Genentech, Inc., except for those for rodent GAPDH which were a generous gift from Perkin Elmer. Reverse transcription was performed at 48 °C for 30 minutes followed by heat activation of AmpliTaq Gold at 95 °C for 10 minutes. Thermal cycling was at 95 °C for 30 seconds and 60 ° for 1.5 minutes for 40 cycles.

Quantitation of the TaqMan results was performed as described by Heid *et al.*, Genome Res. 6:986-994 (1996), with modifications. Briefly, standard curves (1:5 serial dilution) for each target gene of interest were run in duplicate. The Ct was plotted on the Y axis vs the log of the total RNA concentration (X axis), and the equation describing the line was determined. mRNA for each target gene was determined from the appropriate standard curve by entering the Ct (Y value) and solving for the input mRNA (X). The value for the target gene was then normalized to GAPDH by solving the following equation:  $10^{X1}/10^{X2}$ , where X1 is the target gene, and X2 is GAPDH.

## Results

### *IFN- $\gamma$ inhibits cardiac hypertrophy in vivo*

Chronic administration of fluprostenol, an agonist analog of PGF<sub>2 $\alpha$</sub> , has been shown to induce cardiac hypertrophy *in vivo*, and rats with pathologic cardiac hypertrophy induced by myocardial infarction have chronically elevated levels of extractable PGF<sub>2 $\alpha$</sub>  in their myocardium (Lai *et al.*, *supra*). Thus, factors that can inhibit the effects of PGF<sub>2 $\alpha$</sub>  on myocardial growth *in vivo* may be useful for treating cardiac hypertrophy. Rats were dosed with fluprostenol in the presence and absence of IFN- $\gamma$  for two weeks, and the effects on cardiac hypertrophy were determined. Absolute weight of the heart, ventricles, and left ventricle tended to increase in the fluprostenol-treated rats, compared to vehicle controls, and there was significant decrease in these parameters in rats treated with fluprostenol

+ IFN- $\gamma$  relative to fluprostenol treated rats (Table 1). Treatment with fluprostenol resulted in a significant increase in the ratio of heart, ventricular, and left ventricular weights to body weight (BW), indicating that fluprostenol induced cardiac hypertrophy (Figure 4). IFN- $\gamma$  inhibited fluprostenol induced hypertrophy. Rats receiving fluprostenol + IFN- $\gamma$  had significantly decreased heart, ventricular and left ventricular weight, normalized by BW, compared to animals in the fluprostenol groups (Figure 4). Comparison between the IFN- $\gamma$  and vehicle treated groups showed that administration of IFN- $\gamma$  alone did not significantly alter absolute or BW-normalized heart, ventricular, or left ventricular weights (Table 1, Figure 4).

Chronic administration of fluprostenol was associated with a significant decline in mean arterial pressure (MAP) compared to vehicle treated controls (Figure 5). IFN- $\gamma$  had no effect on MAP compared to vehicle, and did not affect the MAP of animals treated with fluprostenol. There was significant alteration in the heart rate in the four treatment groups (Figure 5). These results indicate that IFN- $\gamma$  did not inhibit hypertrophy induced by fluprostenol by counteracting the hemodynamic effects of the treatment.

IFN- $\gamma$  not only inhibited the increase in cardiac mass associated with fluprostenol administration, but also the alterations in cardiac gene expression associated with fluprostenol induced hypertrophy (Figure 6). There was an increase in the abundance of mRNA for  $\alpha$ -skeletal actin, collagen I, and natriuretic factor in the hearts of rats treated with fluprostenol compared to vehicle. The mRNA for sarcoplasmic reticulum calcium ATPase was significantly reduced in these rats. IFN- $\gamma$  inhibited all but the atrial natriuretic factor response.

IFN- $\gamma$  was also tested in a rodent model of cardiac hypertrophy induced by pressure overload generated by abdominal aortic banding. Aortic constriction resulted in cardiac hypertrophy as evidenced by substantial increases in absolute heart, atrial, ventricular and left ventricular weights, and also the ratios of these weights to BW. Treatment with IFN- $\gamma$  significantly attenuated cardiac hypertrophy in this model (Table 2 and Figure 7 and 8).

The effect of IFN- $\gamma$  on other organs was also examined (Table 2). Neither aortic banding nor IFN- $\gamma$  treatment altered kidney weight and the ratio of kidney weight to BW. Compared to sham-operated animals, liver weight and the ratio of BW tended to decrease in aortic-banded rats treated with vehicle, but not in those treated with IFN- $\gamma$ . Aortic constriction caused a significant elevation in absolute and BW-normalized spleen weight, that was exaggerated by IFN- $\gamma$  treatment. Thus, the effects of IFN- $\gamma$  on cardiac mass were not due to a generalized effect on organ weight.

Mean arterial pressure, systolic pressure, and diastolic pressure were markedly higher in rats with aortic constriction compared to sham-operated controls, and the incremental increase in arterial pressure was not different between banded rats treated with IFN- $\gamma$  or vehicle (Figure 9). This result indicates that the attenuation of cardiac hypertrophy observed in banded rats receiving IFN- $\gamma$  did not relate to an alteration in afterload.

Aortic constriction resulted in several changes in cardiac gene expression. The relative abundance of mRNA for  $\beta$ -myosin heavy chain,  $\alpha$ -smooth muscle and  $\alpha$ -skeletal actins, atrial natriuretic factor, collagens I and III, and



fibronectin were all increased in banded rats compared to sham-operated controls. The effects on two of these genes;  $\alpha$ -smooth muscle actin and collagen I were inhibited by IFN- $\gamma$  (Table 3).

Taken together, the results in Examples 1 and 2 show that IFN- $\gamma$  can inhibit cardia hypertrophy. The effects of IFN- $\gamma$  are not limited to inhibiting an increase in cardiac mass induced by hypertrophic stimuli, IFN- $\gamma$  can also inhibit certain of the molecular alterations that occur in the hypertrophied heart at the level of gene expression. It is especially noteworthy that IFN- $\gamma$  inhibited the induction of collagen I gene expression *in vivo*, both in response to chronic stimulation with fluprostenol and in a model of hypertrophy induced by pressure overload. Collagen I accounts for approximately 75% of myocardial collagen (Ju *et al.*, *Can. J. Cardiol.* 12:1259-1267 [1996]). Increased extracellular matrix deposition and interstitial fibrosis that accompany cardiac hypertrophy can contribute to the pathophysiology of heart failure. By inhibiting collagen I production, IFN- $\gamma$  may reduce interstitial fibrosis in the setting of heart failure.

**Table 1. Body Weight and Organ Weight  
in Rats Treated with Flup and/or IFN**

	Vehicle	Flup	Flup+IFN	IFN
BWO (g)	292.4 $\pm$ 1.7	292.3 $\pm$ 2.2	292.8 $\pm$ 2.1	292.5 $\pm$ 3.2
BW (g)	391.6 $\pm$ 6.3	381.1 $\pm$ 4.4	377.6 $\pm$ 4.5	380.8 $\pm$ 6.1
$\Delta$ BW (g)	99.2 $\pm$ 5.5	91.9 $\pm$ 4.0	84.8 $\pm$ 3.9	88.3 $\pm$ 5.0
HW (g)	.966 $\pm$ .022	1.000 $\pm$ .018	.9279 $\pm$ .016#	.956 $\pm$ .029
VW (g)	.922 $\pm$ .022	.957 $\pm$ .017	.889 $\pm$ .015#	.914 $\pm$ .029
LVW (g)	.706 $\pm$ .018	.740 $\pm$ .013	.678 $\pm$ .011##	.696 $\pm$ .023
KW (g)	1.440 $\pm$ .035	1.397 $\pm$ .038	1.377 $\pm$ .031	1.327 $\pm$ .035*
KW/BW (g/kg)	3.678 $\pm$ .075	3.632 $\pm$ .076	3.648 $\pm$ .070	3.483 $\pm$ .069
SW (g)	.799 $\pm$ .050	.880 $\pm$ .048	1.009 $\pm$ .042*	.924 $\pm$ .068
SW/BW (g/kg)	2.065 $\pm$ .149	2.309 $\pm$ .130	2.676 $\pm$ .082**	2.415 $\pm$ .149

Data expressed as mean $\pm$ SEM, and animal numbers are 14, 14, 14, and 9 in the Vehicle, Flup, Flup+IFN, and IFN group, respectively. Vehicle, saline; Flup, fluprostenol; IFN, interferon  $\gamma$ , BWO, basal levels of body weight; BW, body weight post treatment;  $\Delta$ BW, BW-BWO; HW, heart weight; VW, ventricular weight; LVW, left ventricular weight; KW,

kidney weight; SW, spleen weight. \* $p<0.05$ , \*\* $P<0.01$ , compared to the Vehicle group, # $p<0.05$ , ## $p<0.01$ , compared to the Flup group.

**Table 2. Body Weight, Organ Weight,  
and HR in Rats with Pressure Overload**

	Sham	PO+vehicle	PO+IFN
BWO (g)	278.8±1.9	279.4±1.3	279.0±1.3
BW (g)	367.9±6.8	347.5±5.7*	355.5±5.2
ΔBW (g)	89.1±5.8	68.1±5.6*	76.5±4.7
AW (g)	.038±.002	.056±.002**	.046±.003*##
AW/BW (g/kg)	.104±.004	.162±.006**	.129±.007*##
KW (g)	1.438±.051	1.334±.033	1.349±.071
KWBW (g/kg)	3.894±.078	3.841±.070	3.776±.071
LW (g)	13.84±.55	12.53±.36	13.96±.47#
LW/BW (g/kg)	37.46±.96	36.01±.71	38.94±.92#
SW (g)	.724±.030	.839±.026*	1.170±.053***
SW/BW (g/kg)	1.959±.051	2.418±.069**	3.261±.121***
HR (bpm)	371±12	415±12*	418±19*

Data expressed as mean±SEM. Animal numbers are 16, 22, and 21 in the Sham, PO+vehicle, and PO+IFN group, respectively, for all parameters except HR for which animal number are 7, 8, and 7, respectively. PO, pressure overload; IFN, interferon  $\gamma$ ; BWO, basal levels of body weight; BW, body weight post treatment; ΔBW, BW-BWO; AW, atrial weight; KW, kidney weight; LW, liver weight; SW, spleen weight; HR, heart rate. \* $p<0.05$ , \*\* $P<0.01$ , compared to the sham group. # $p<0.05$ , ## $p<0.01$ , compared to the PO+vehicle group.

**Table 3. Effect of IFN on Gene Expression**

Treatment	SHAM + Vehicle	PO + Vehicle	PO + IFN
ANF	0.98±0.37	5.29±1.21†	3.61±1.32
βMHC	0.89±0.24	1.91±0.15†	1.71±0.14†
SKA	0.95±0.13	3.35±0.46†	2.47±0.51†
SMA	0.71±0.06	0.89±0.04†	0.77±0.06
COLI	0.55±0.05	0.91±0.09†	0.77±0.10
COLIII	0.44±0.05	0.66±0.08†	0.72±0.09†
FIB	0.66±0.17	1.03±0.11†	0.97±0.12

PO indicates pressure overload; IFN, interferon-gamma; ANF, atrial natriuretic factor; βMHC, β-myosin heavy chain; SKA, α-skeletal actin; SMA, α-smooth muscle actin; COLI, collagen I; COLIII, collagen III; FIB, fibronectin. Expression levels are calculated as ratios to glyceraldehyde-3-phosphate dehydrogenase. n=6 per group. Values are mean±SEM. †P<0.05 vs sham+vehicle group.

†

**Claims:**

1. A method of treating cardiac hypertrophy comprising administering to a patient having cardiac hypertrophy a therapeutically effective amount of interferon gamma (IFN- $\gamma$ ).
2. The method of claim 1 wherein said patient is human.
3. The method of claim 2 wherein said IFN- $\gamma$  is recombinant human IFN- $\gamma$ (rh-IFN- $\gamma$ ).
4. The method of claim 3 wherein said IFN- $\gamma$  is rhIFN- $\gamma$ -1b.
5. The method of claim 3 wherein said cardiac hypertrophy is characterized by the presence of an elevated level of PGF<sub>2 $\alpha$</sub> .
6. The method of claim 2 wherein said cardiac hypertrophy has been induced by myocardial infarction.
7. The method of claim 6 wherein said IFN- $\gamma$  administration is initiated within 48 hours following myocardial infarction.
8. The method of claim 7 wherein said IFN- $\gamma$  administration is initiated within 24 hours following myocardial infarction.
9. The method of claim 2 wherein said patient is at risk of developing cardiac hypertrophy.
10. The method of claim 9 wherein said patient has suffered myocardial infarction.
11. The method of claim 10 wherein said IFN- $\gamma$  administration is initiated within 48 hours following myocardial infarction.
12. The method of claim 11 wherein said IFN- $\gamma$  administration is initiated within 24 hours following myocardial infarction.

13. The method of claim 2 wherein said IFN- $\gamma$  is administered in combination with at least one further therapeutic agent used for the treatment of cardiac hypertrophy or a heart disease resulting in cardiac hypertrophy.

14. The method of claim 13 wherein said further therapeutic agent is selected from the group consisting of  $\beta$ -adrenergic-blocking agents, verapamil, diltiazem, and diltiazem.

15. The method of claim 14 wherein said  $\beta$ -adrenergic blocking agent is carvedilol, propranolol, metoprolol, timolol, oxprenolol or tertatolol.

16. The method of claim 13 wherein said IFN- $\gamma$  is administered in combination with an antihypertensive drug.

17. The method of claim 13 wherein said IFN- $\gamma$  is administered with an ACE-inhibitor.

18. The method of claim 13 wherein said IFN- $\gamma$  is administered with an endosthelin receptor antagonist.

19. The method of claim 13 wherein said IFN- $\gamma$  is administered following the administration of a thrombolytic agent.

20. The method of claim 18 wherein said thrombolytic agent is recombinant human tissue plasminogen activator (rht-PA).

21. The method of claim 13 wherein said IFN- $\gamma$  is administered following primary angioplasty for the treatment of acute myocardial infarction.

22. A method for making a pharmaceutical composition for the treatment of cardiac hypertrophy, comprising admixing a therapeutically effective amount of interferon gamma (IFN- $\gamma$ ) with a pharmaceutically acceptable carrier.

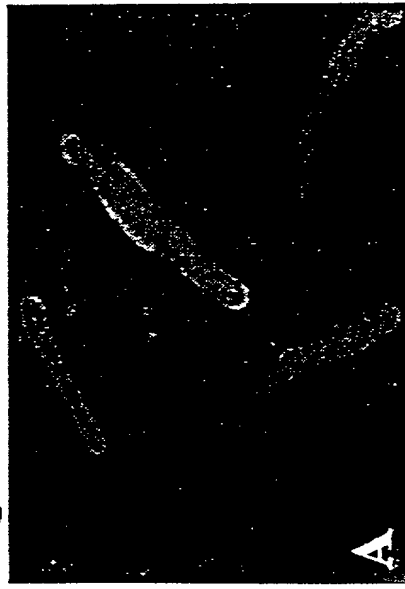
23. The method of claim 21 wherein said pharmaceutical composition is liquid.

24. The method of claim 22 wherein said pharmaceutical composition comprises a preservative.
25. The method of claim 23 wherein said pharmaceutical composition is an injectable formulation.
26. A pharmaceutical product comprising:
  - (a) a pharmaceutical composition comprising at least one therapeutically effective dosage of IFN- $\gamma$ ;
  - (b) a container containing said pharmaceutical composition; and
  - (c) a label affixed to said container, or a package insert included in said pharmaceutical product referring to the use of said IFN- $\gamma$  in the treatment of cardiac hypertrophy.
27. The pharmaceutical product of claim 25, wherein said container has a sterile access port.
28. The pharmaceutical product of claim 26 wherein said container is an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

## Abstract of the Disclosure

The invention concerns the treatment of cardiac hypertrophy by interferon-gamma (IFN- $\gamma$ ). Cardiac hypertrophy may result from a variety of diverse pathologic conditions, including myocardial infarction, hypertension, hypertrophic cardiomyopathy, and valvular regurgitation. The treatment extends to all stages of the progression of cardiac hypertrophy, with or without structural damage of the heart muscle, regardless of the underlying cardiac disorder.

**Figure 1**



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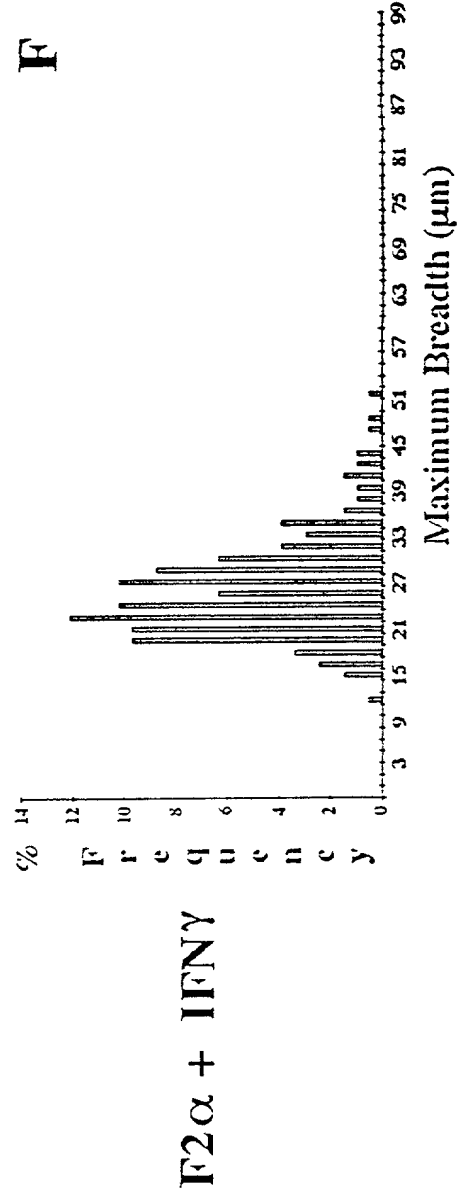
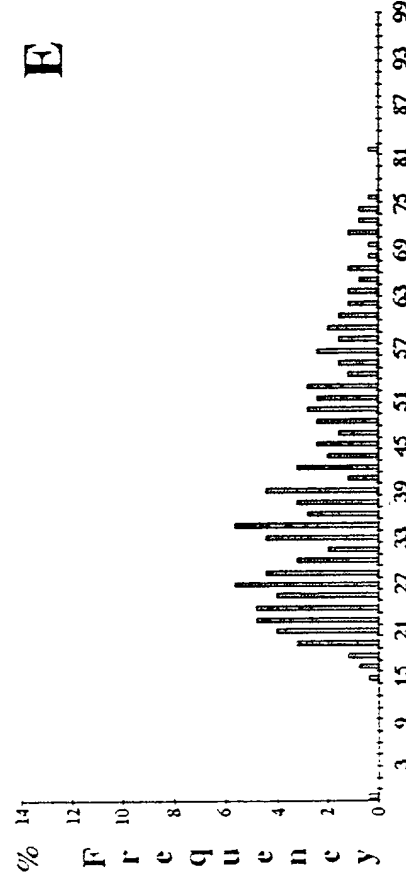
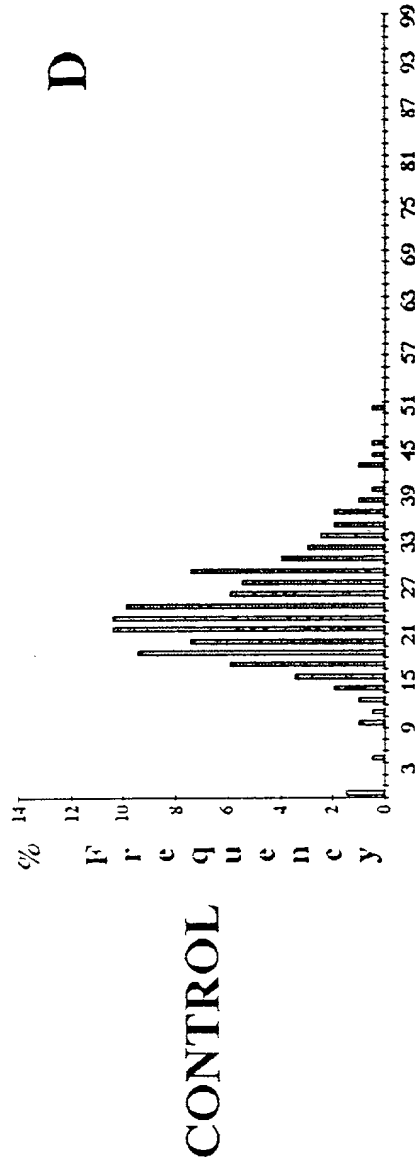




Figure 2

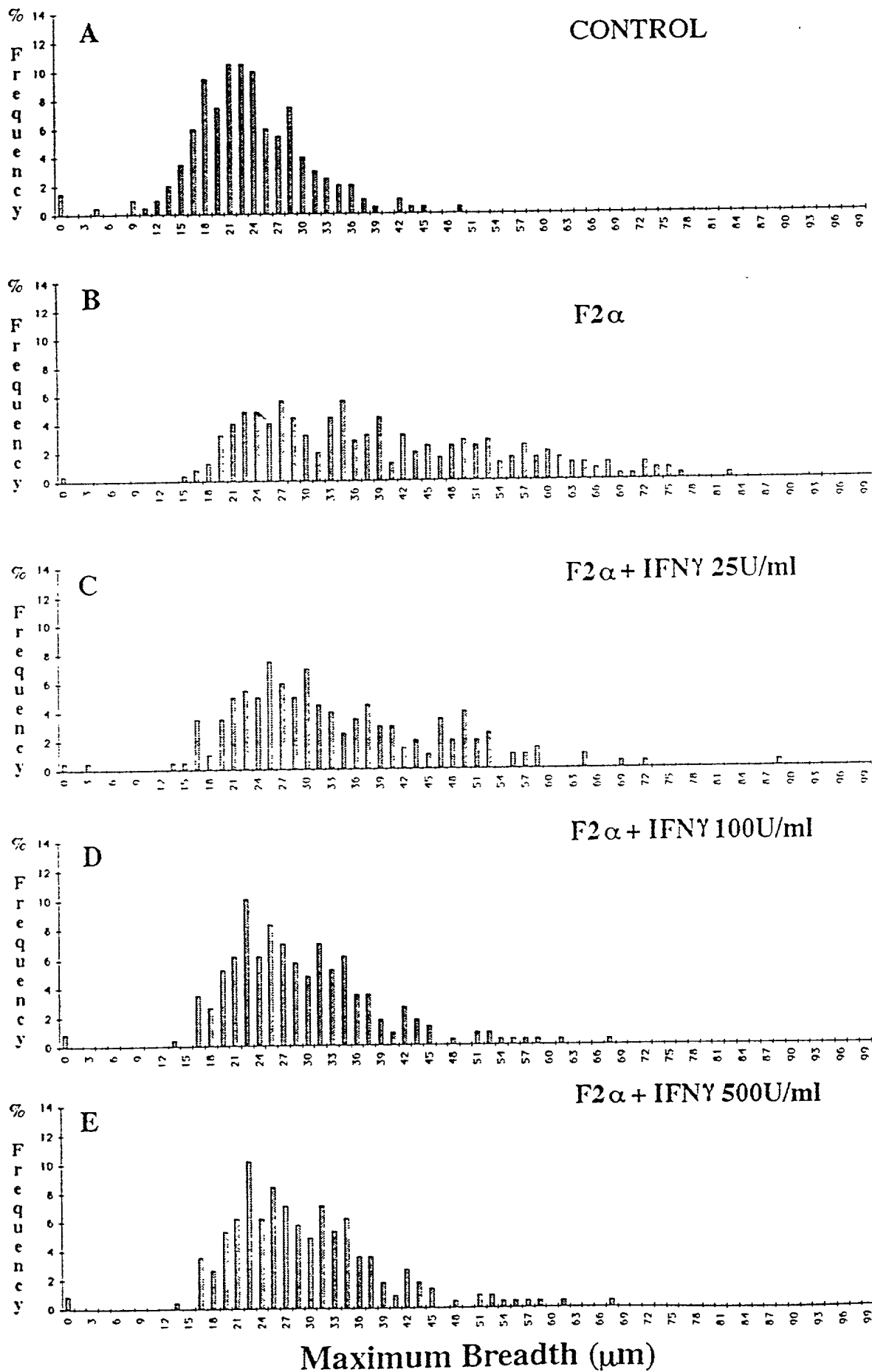


Figure 3

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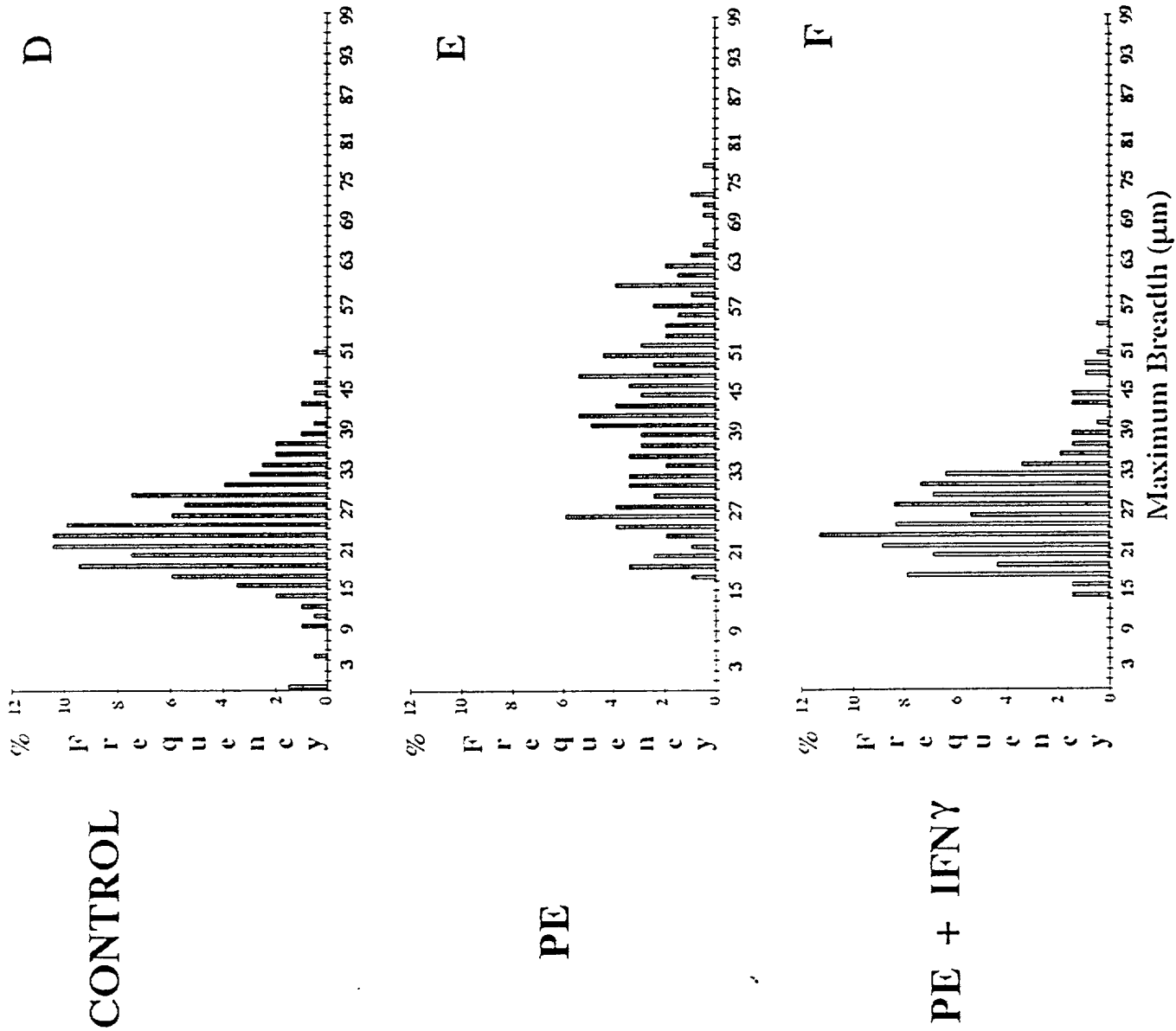
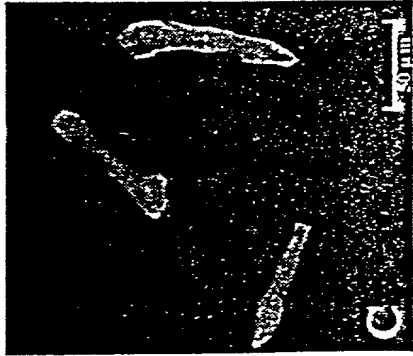
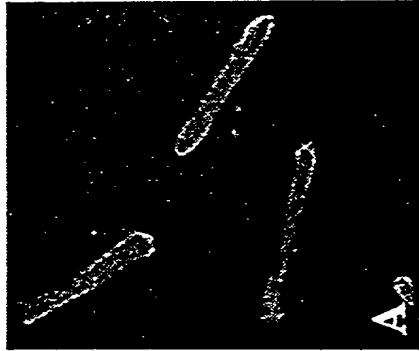


FIGURE 4

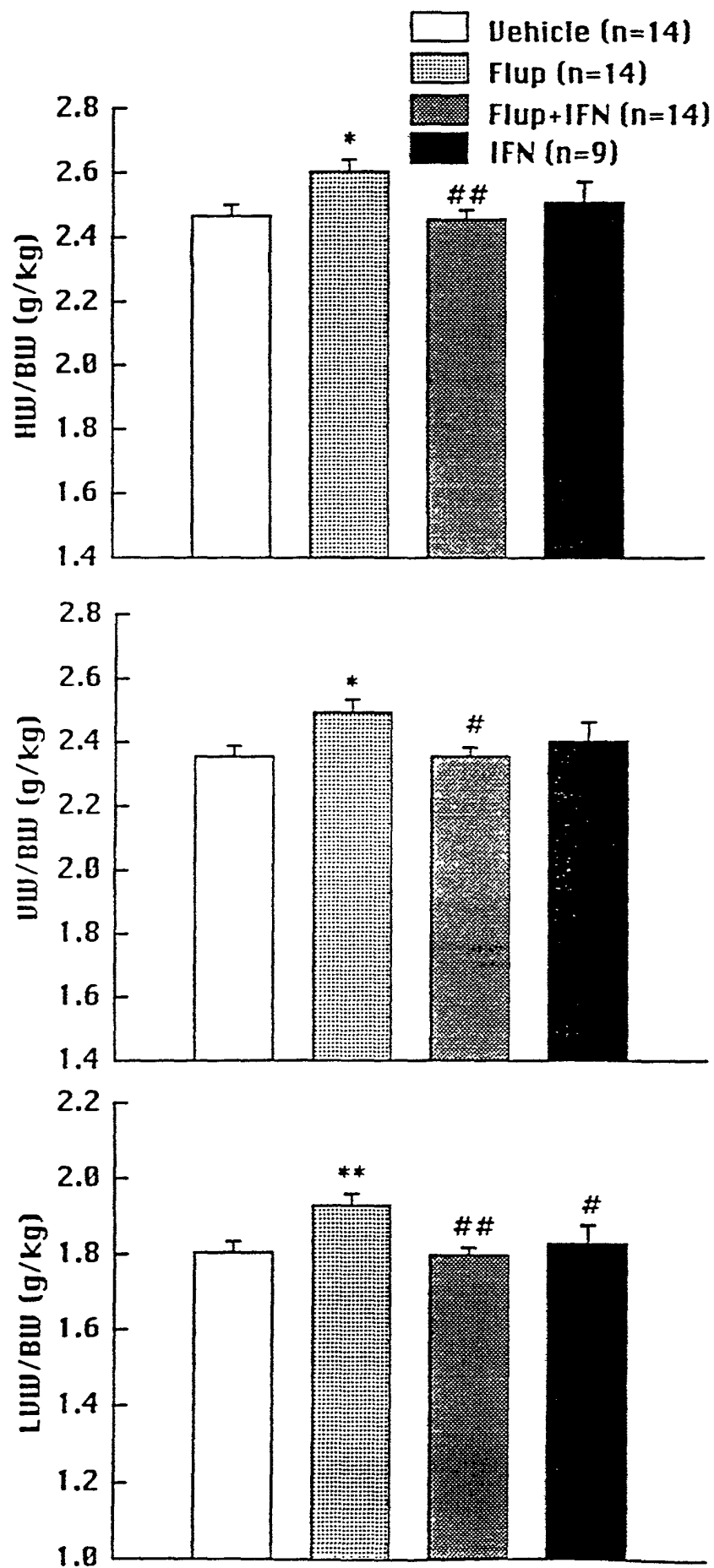
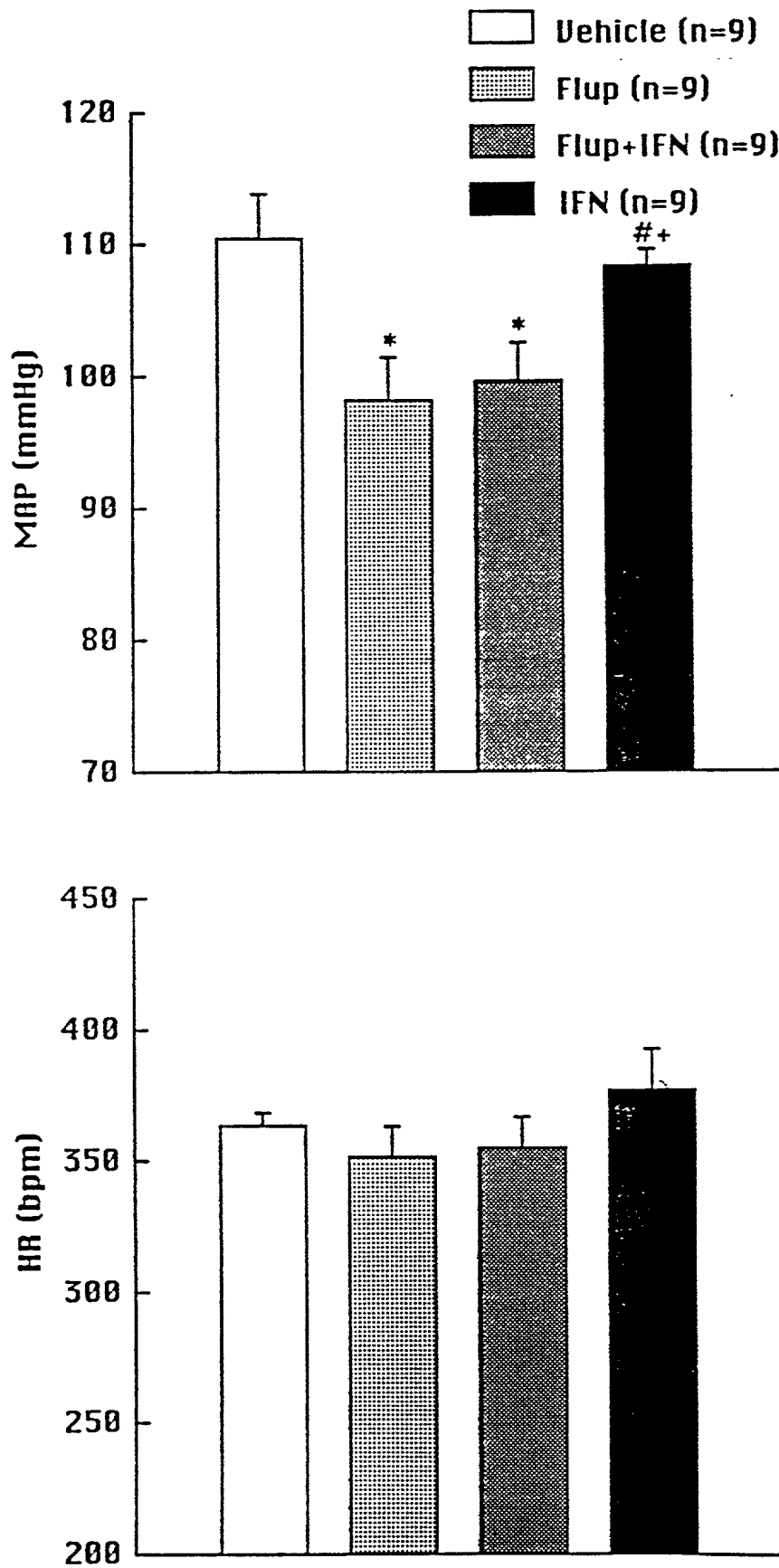


FIGURE 5



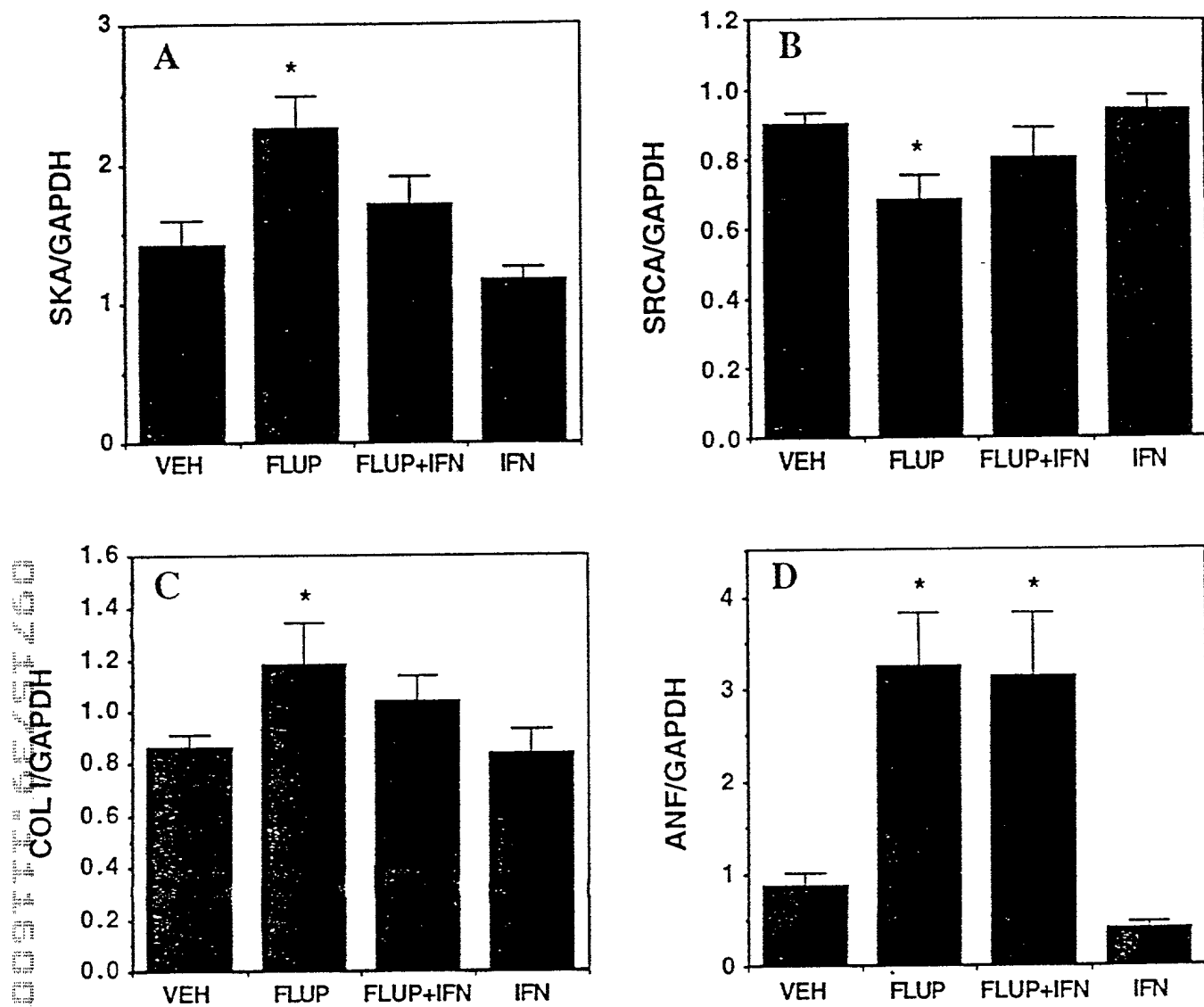


FIGURE 6

FIGURE 7

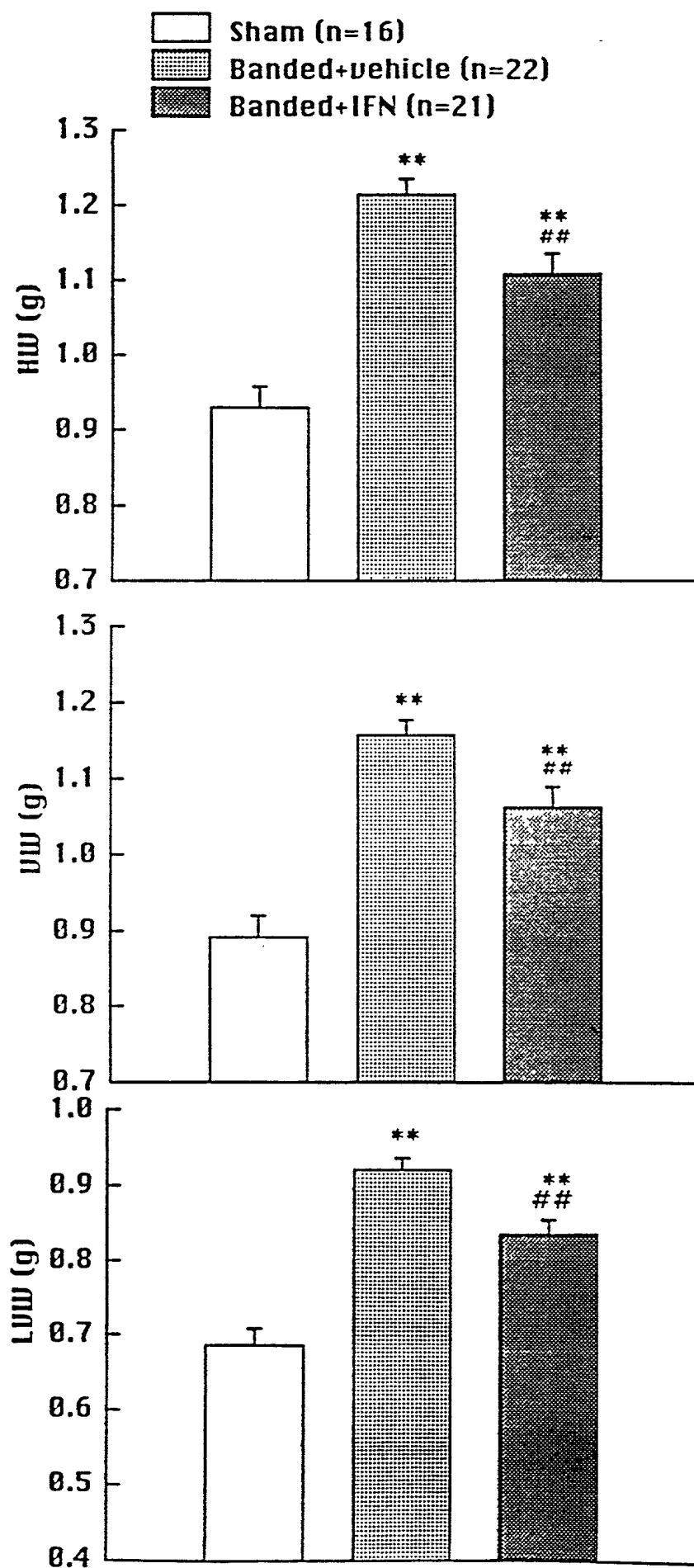


FIGURE 8

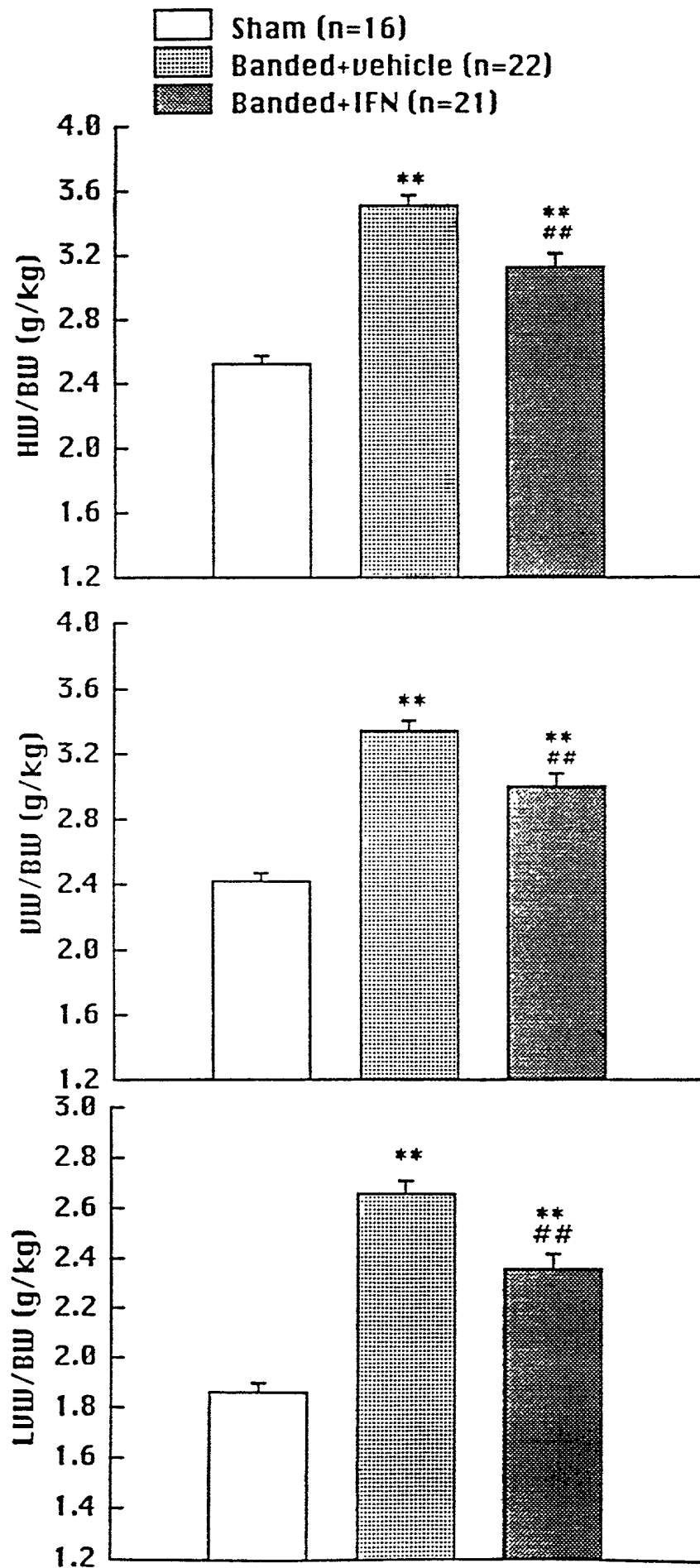
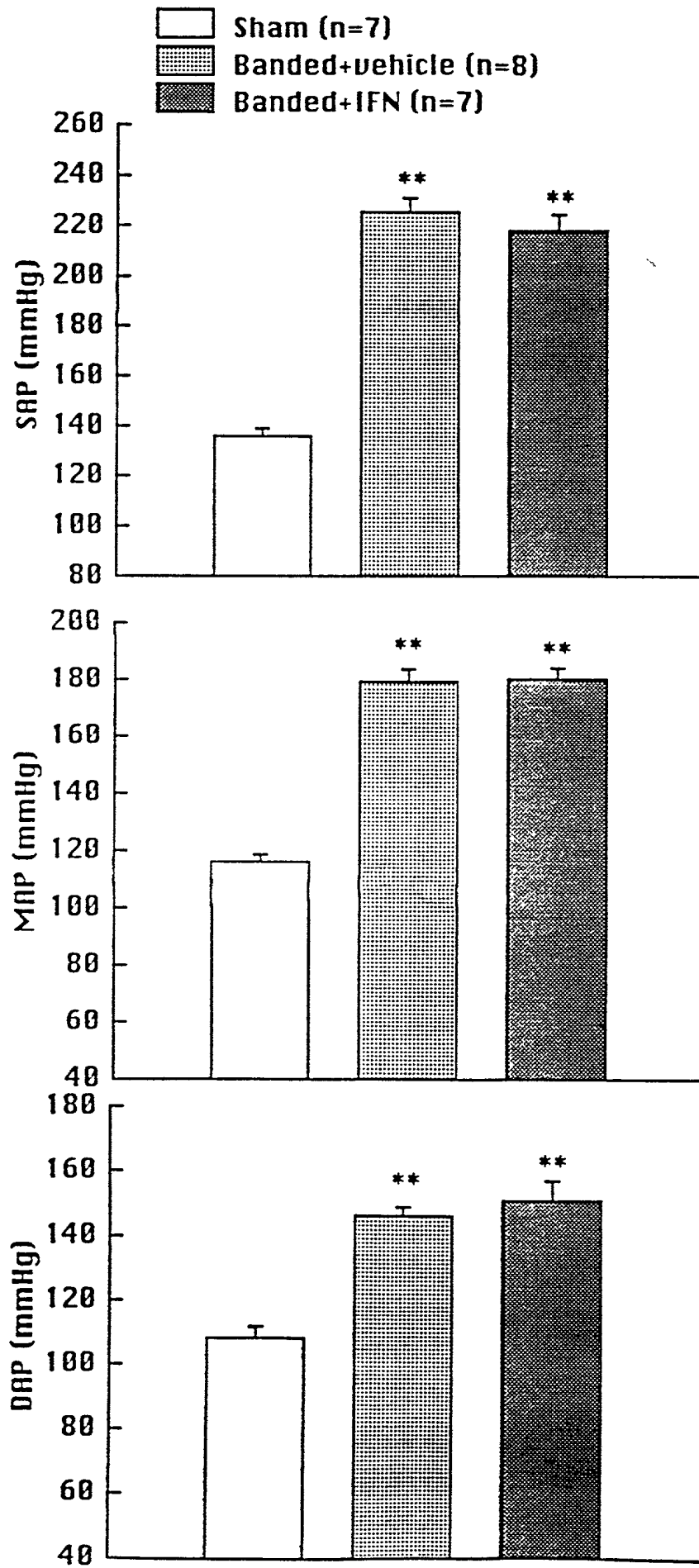


FIGURE 9





**COMBINED DECLARATION FOR PATENT APPLICATION  
AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**EFFECTS OF IFN- $\gamma$  ON CARDIAC HYPERTROPHY**

the specification of which (check one) ☐ is attached hereto or ☒ was filed on March 19, 1999 as Application Serial No. 09/273,099 and was amended on    (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):

Priority Claimed

Yes      No

Number	Country	Day/Month/Year Filed
I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:		
60/080,448		2 April 1998
Application Ser. No.		Filing Date
I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:		
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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	Yes      No

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## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Hongkui Jin et al. ) Group Art Unit: 1646  
App. No. : 09/273,099 ) I hereby certify that this correspondence and all  
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)  
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)

ASSOCIATE POWER OF ATTORNEY AND CHANGE OF ADDRESS

Assistant Commissioner for Patents  
Washington, D.C. 20231

Dear Sir:

The attorneys of the law firm of Knobbe, Martens, Olson & Bear, **Customer No. 20,995**, are hereby appointed associate Powers of attorney to prosecute the above-identified application and take all actions respecting the same. **Please use Customer No. 20,995** for all communications.

Please also send all communications to the following address:

GINGER R. DREGER  
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620 Newport Center Drive  
Sixteenth Floor  
Newport Beach, CA 92660  
(949) 760-0404

Application No.: 09/273,099

Filed: March 19, 1999

Please charge the new firm's deposit account number 11-1410, for any fees which may be due or outstanding in connection with this case.

Respectfully submitted,

GENENTECH, INC.

Dated: 28 May 2000

By: 

Rick B. Love

Registration No. 34,659

Attorney of Record

1 DNA Way

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